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Progesterone promotes the development and growth of breast cancers. Inhibitors of progesterone are in clinical trials for the treatment of breast cancers. A problem with all endocrine therapies is that the tumors eventually become resistant. We propose to study the molecular biology of antiprogestins in breast cancer, focusing on mechanisms by which their inhibitory actions are inappropriately switched to stimulatory ones. Antiprogestins regulate gene transcription by binding to progesterone receptors (PR). Human breast cancer cells contain mixtures of two PR -- truncated A-receptors, and B-receptors which have an N-terminal extension (the B-upstream segment, BUS). A- and B-receptors are functionally dissimilar, and antagonist-occupied B-receptors (but not A-receptors) stimulate transcription inappropriately. 1) We have constructed new breast cancer cell lines that contain only A- or B-receptors. These have been used to study isoform-dependent growth regulation, and to explain why progesterone can regulate both cell growth and differentiation. We have analyzed the mechanisms involved in resistance to the growth regulatory effects of progesterone. 2) Studies dealing with the role of phosphorylation on DNA binding and transcriptional functions of PR, focus on BUS. 3) We have discovered a novel inhibitory function (IF) that works only in the context of A-receptors.

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(4) TABLE OF CONTENTS

(5) INTRODUCTION

Nature of the Problem and Background:

Women develop breast cancer because they have ovaries. Analysis of factors involved in breast cancer induction, growth, and treatment has therefore focused on estradiol and progesterone; particularly on estradiol. The latter is a growth promoter, and blockade of estradiol at the tumor with the antiestrogen tamoxifen is a major thrust of current endocrine therapies. But is estradiol the only ovarian hormone with an impact on breast cancer development and proliferation? Progesterone has a proliferative effect in the epithelium of the normal breast, and progestin agonists at physiological doses promote the growth of experimental mammary cancers. Progesterone antagonists inhibit the growth of breast cancer (1-3).

In human breast cancers there are two, naturally occurring, PR isoforms: the 769 amino acid A-receptors; and the 933 amino acid B-receptors, that have a 164 amino acid extension at the N-terminus -- which we call the B-upstream segment, or BUS. Downstream of BUS, A- and B-receptors are identical. The two receptors can form homo- and heterodimers, leading to three dimeric species (A:A, A:B, B:B) that bind DNA (1, and references therein). Our preliminary data show that the A- and B-isoforms are functionally dissimilar when occupied by agonists. Additional data show that progesterone antagonists can either inhibit or stimulate transcription, depending on the receptor isoform, the promoter of the gene being regulated, and the modulatory influence of other signalling pathways, particularly of cAMP (4,5). **We postulate that breast cancers become "resistant" to hormone therapy because antagonists acquire inappropriate, agonist-like, effects. The clinical consequences of such a functional switch are grave. The studies we have proposed address the molecular mechanism by which antagonist-occupied progesterone B-receptors become transcriptional agonists.**

Purpose of Present Work and Methods of Approach:

Aim 1. The functional differences between A- and B-receptors in breast cancer cells. B- and A-receptors are present together in breast cancers. Transient transfection methods show that antagonist-occupied B-receptors, but not A-receptors, can act like transcriptional agonists in a promoter-specific manner or when cAMP levels are increased. We plan to stably transfect PR-negative breast cancer cell lines with expression vectors encoding B- or A-receptors. This allows each receptor isoform to be studied independently while being expressed at normal levels in a physiological setting. The cells will be used to assess long-term growth effects, and transcriptional regulation, by progesterone agonists and antagonists, with or without increased cAMP levels. For transcriptional studies, different promoter-reporter constructs will be analyzed. These studies will define the effects of each receptor isoform on the functional end-points of transcription and cell proliferation in the physiologically relevant setting of breast cells.

Aim 2. BUS - The B-upstream segment. A third transcriptional activation domain unique to B-receptors? Since only PR B-receptors anomalously induce transcription in the presence of progesterone antagonists, we will focus on their unique 164 amino acid extension -- the B-

upstream segment, or BUS. Preliminary data show that BUS contains a novel transcriptional activation function we call AF-3. This is in addition to two other AFs (AF-1 and AF-2) that are common to both receptor isoforms. We postulate that AF-3 functions by binding one or more coactivator proteins. We will construct a series of expression vectors of BUS alone, or of BUS fused to the DNA binding domain (DBD) and nuclear localization signal (NLS) of PR. These constructs will be tested for their ability to constitutively regulate transcription; to "squelch" full-length B-receptor actions; to complement A-receptors; and to be cAMP modulated, all in promoter and cell-specific contexts. Mutants will be constructed of 5 *ser-pro* clusters found on BUS, in order to map AF-3, and to analyze the control of B-specific transcription by phosphorylation. The studies in this aim will functionally define and characterize AF-3; a site unique to B-receptors.

Aim 3. **Mechanisms of AF-3 action in the BUS segment.** Antagonist-occupied B-receptors activate transcription in a promoter-specific fashion: on the mouse mammary tumor virus (MMTV) promoter when cAMP levels are elevated; on the *Herpes simplex* virus thymidine kinase (*tk*) promoter, through a novel, PRE-independent mechanism. First, we plan to identify *cis*-acting elements on the MMTV and *tk* promoters through which antagonist-occupied B-receptors stimulate transcription. Site-specific mutants of the MMTV promoter will seek the *cis*-acting elements that eliminate cAMP effects without loss of PR-regulated transcription. We will test the hypothesis that cAMP acts through novel DNA elements that cooperatively bind the ATF/CREB and HMG family of proteins, and interact with BUS. Linker-scanning mutants of the *tk* promoter will be used to define elements that mediate antagonist-occupied B-receptor stimulation of transcription. Second, protein-protein interactions between antagonist-occupied B-receptors and as yet unknown coactivators will be characterized using bacterially produced BUS fusion proteins, or the yeast two-hybrid system to identify, isolate and clone cDNAs encoding nuclear proteins that interact with BUS and to analyze their tissue-specific distribution. The studies in this aim will define novel coactivator proteins that interact with B receptor isoform of PR, and select their direction of transcription.

(6) BODY

Three papers related to these aims have been published or are in preparation:

a) Groshong SD, Schauer IE, Todd LM, Langan TA, Sclafani RA and **Horwitz KB**. A cell cycle restriction point controlled by progesterone sensitizes breast cancer cells to cross-talk with epidermal growth factor. Submitted, 1996. See manuscript appended.

We have now carried out an extensive series of studies related to Aim 1, using the newly constructed cell lines T47D-YA and T47D-YB, to explain the role of progestins on the growth of breast cancers.

Depending on the tissue, progesterone is either a proliferative or a differentiative hormone. To explain this paradox, and to simplify analysis of the effects of progesterone, we used our recently developed sublines (T47D-YB) of the estrogen resistant and epidermal growth factor (EGF) resistant $T47D_{CO}$ breast cancer cell line that constitutively express the B-isoform of progesterone receptors (PR). Progesterone treatment accelerates T47D-YB cells through the first mitotic cell cycle, but arrests them at the G1/S boundary of the second cycle. Additional progesterone cannot restart cell proliferation despite adequate levels of transcriptionally competent PR. The profound progesterone resistance is accompanied by decreased levels of cyclins D1, D3 and E, disappearance of cyclins A and B, a transient 5-fold induction of the cdk inhibitor p21, and a sustained 5-fold increase in the levels of $p27^{Kip1}$. During the resistant phase, $p27^{Kip}$ levels rise even higher after additional progesterone treatment. Further, the retinoblastoma protein product is hypophosphorylated and extensively down-regulated. However, despite absolute progesterone resistance, the cell cycling machinery is poised to restart, and does so rapidly in response to EGF, during a narrow time interval in which cdk inhibition levels are depressed. Thus progesterone pretreatment sensitizes the cells to EGF. We propose that progesterone is neither inherently proliferative nor antiproliferative, but that it controls a cell-cycle competency point, upon which other tissue-specific factors like EGF, influence the fate of the cell.

We believe that these data are very exciting since they explain for the first time, why a hormone like progesterone can have different effects on growth in two target tissues -- like the uterus vs. the breast. The data also, for the first time, begin to explain why development of resistance to steroid hormones in breast cancer may lead to enhanced sensitivity to growth factors in that cancer, resulting in accelerated cancer growth. Studies are in progress to follow-up on these findings.

b) Takimoto GS, Tung L, Hovland AR, Powell RL and **Horwitz KB**. Role of phosphorylation on DNA binding and transcriptional functions of human progesterone receptors. *J Biol Chem* 271:13308-13316, 1996. See reprint appended.

This paper deals with issues raised in Aim 2.

To study the function of human hPR phosphorylation focusing on BUS, we have tested four sets of serine to alanine substitution mutants: 10 serine clusters, located in regions common

to both hPR isoforms (the M-series mutants) were mutated in 1) A-receptors and 2) B-receptors; 6 serine clusters located in the B-upstream segment (BUS; the B-series mutants) were mutated individually and collectively and cloned into 3) B-receptors and 4) into BUS-DBD-NLS, a constitutive transactivator, in which the AF3 function of BUS is fused to the DNA binding domain (DBD) and nuclear localization signal (NLS) of hPR. Transcription by most of the M-series mutants resembles that of wild-type A- or B-receptors. Mutation of three sites -- Ser^{190} at the N-terminus of A-receptors; a cluster of serines just upstream of the DBD; or Ser^{676} in the hinge region -- inhibits transcription by 20-50% depending on cell or promoter context. These sites lie outside the AF1 activation function. M-series mutants are substrates for a hormone-dependent phosphorylation step and they all bind well to DNA. Progressive mutation of the B-series clusters leads to the gradual dephosphorylation of BUS, but only the 6-site mutant, involving 10 serine residues, is completely dephosphorylated. These data suggest that in BUS, alternate serines are phosphorylated or dephosphorylated at any time. However, even when BUS is completely dephosphorylated, both BUS-DBD-NLS and full-length B-receptors remain strong transactivators. Mutant B-receptors also do not acquire the dominant negative properties of A-receptors, and they retain the ability to activate transcription in synergy with 8-Br-cAMP and antiprogestins. We conclude that phosphorylation has subtle effects on the complex transcriptional repertoire that distinguishes the two hPR isoforms and does not influence transactivation mediated by AF1 or AF3, but subserves other functions.

We now have preliminary data that phosphorylation of a site in IF (see below) strongly influences BUS activity, and we are beginning to test the hypothesis that intramolecular contacts formed between BUS and "phospho" IF are responsible for the strong biological activity of B-receptors.

c) Hovland AR, Powell RL, Takimoto GS, Tung L and **Horwitz KB**. A novel N-terminal inhibitory function (IF) and three activation functions (AFs) in human progesterone A- and B-receptors control their transcriptional diversity. *J Biol Chem*, under revision, 1996. See manuscript appended.

This paper also deals with issues raised in Aim 2.

The B-isoform of human progesterone receptors (PR) contains three activation functions (AF3, AF1, AF2), two of which (AF1, AF2) are shared with the A-receptor isoform. AF3 is in the B-upstream segment (BUS) at the far N-terminal 164 amino acids of B-receptors, AF1 is in the 392 amino acid N-terminal region common to both receptors, and AF2 is in the hormone binding domain. To study the questions raised in Aim 1 we have shown that cooperativity among these AFs accounts for promoter and cell-specific differences in the transcriptional activity of the two receptors. On two promoters -- the natural promoter of the mouse mammary tumor virus LTR, and a synthetic promoter that contains two progesterone response elements -- B-receptors have five to ten-fold greater transcriptional activity than A-receptors. This is due to transcriptional synergy produced by cooperativity between AF3, and one of the two downstream AFs. Besides cooperative activity, each AF exhibits autonomous transactivating capacity when it is linked alone to the DNA binding domain of PR. The extent of this transcription is, however, promoter- and cell-specific, and is generally higher in HeLa cervicocarcinoma cells, than in T47D

breast cancer cells. In addition to transactivating activity, we find that the N-terminus of PR common to both isoforms, contains an inhibitory function (IF), located in a 292 amino acid segment lying between AF3 and AF1. IF suppresses the activity of A-receptors, but is not inhibitory in the context of B-receptors, due perhaps to activating contacts formed between "phospho" IF and BUS (see "a)" above). As a result, IF inhibits AF1 or AF2, but not AF3. These data demonstrate the existence of a novel inhibitory function in PR, which, together with the three AFs, accounts in part for the complex transcriptional repertoire of these receptors. Furthermore, mapping of IF to the N-terminus begins to assign novel functions to this large, relatively undefined, structural region of human PR.

We now believe that intramolecular contacts formed between BUS and IF create the active surface that explains the strong activity of B-receptors (see Aim 3).

d) To address mechanisms of AF3 action raised in Aim 3 we have used $LexA_{DBD}$ BUS and $LexA_{DBD}$ IF as independent baits in a yeast protein interaction screen to search for nuclear coactivators or corepressors that bind to these PR domains and are responsible for their transcriptional phenotype. Using a promoter with five LexA DNA binding sites we find that both BUS and IF have autonomous transcriptional activity making a protein-protein interaction screen by this method impossible. We have now obtained a promoter carrying only one LexA DNA binding site which may mute the constitutive activity of the two chimeric PR bait constructs. We are currently rescreening both. However, based on the results described in b) and c) above, we now believe that folding of BUS over "phospho" IF creates the transcriptionally active surface, and we may have to reconstruct our bait with this in mind.

Nevertheless, while screening with a BUS-DBD construct (i.e. with AF3 alone) we discovered a novel protein complex that binds to the PR DBD and has strong DNA-dependent protein kinase activity. Studies designed to extend these observations are in progress.

(7) CONCLUSIONS

This year's studies have allowed us to progress in all three of our original aims. Note however that the new insights we have gained will lead us in directions that were not completely forseen in the original grant. For example, we plan to expand our understanding of the cross-talk between PR and EGF by focusing on the role of mitogen activated protein (MAP) kinase activity (rather than on cAMP) since a) EGF activates the MAP kinase pathway, and b) the phosphorylation site on IF that activates B-receptors is a MAP kinase consensus site. Additionally, we plan to follow-up on characterization of the multi-protein complex that binds to the PR-DBD -- an observation that was not anticipated in the original aims. Finally, we will revise our protein-protein screening strategy for nuclear coregulatory factors that might bind to BUS (AF3), by using a BUS-"phospho" IF construct as bait, together with less sensitive yeast reporter plasmids.

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(9) APPENDIX

Copy of manuscript submitted, Groshong SD *et al*, 1996
Reprint of Takimoto GS *et al*, *J Biol Chem* 271:13308-13316, 1996
Copy of manuscript under revision, Hovland AR *et al*, 1996

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Role of Phosphorylation on DNA Binding and Transcriptional Functions of Human Progesterone Receptors*

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To study the function of human progesterone receptor (hPR) phosphorylation, we have tested four sets of serine to alanine substitution mutants: 10 serine clusters, located in regions common to both hPR isoforms (the M-series mutants) were mutated in A-receptors and B-receptors; 6 serine clusters located in the B-upstream segment (BUS; the B-series mutants) were mutated individually and collectively and cloned into B-receptors and into BUS-DBD-NLS, a constitutive transactivator, in which the AF3 function of BUS is fused to the DNA binding domain (DBD) and nuclear localization signal (NLS) of hPR. Transcription by most of the M-series mutants resembles that of wild-type A- or B-receptors. Mutation of 3 sites, Ser^{190} at the N terminus of A-receptors, a cluster of serines just upstream of the DBD, or Ser^{676} in the hinge region, inhibits transcription by 20–50% depending on cell or promoter context. These sites lie outside the AF1 activation function. M-series mutants are substrates for a hormone-dependent phosphorylation step, and they all bind well to DNA. Progressive mutation of the B-series clusters leads to the gradual dephosphorylation of BUS, but only the 6-site mutant, involving 10 serine residues, is completely dephosphorylated. These data suggest that in BUS alternate serines are phosphorylated or dephosphorylated at any time. However, even when BUS is completely dephosphorylated, both BUS-DBD-NLS and full-length B-receptors remain strong transactivators. Mutant B-receptors also do not acquire the dominant negative properties of A-receptors, and they retain the ability to activate transcription in synergy with 8-Br-cAMP and antiprogestins. We conclude that phosphorylation has subtle effects on the complex transcriptional repertoire that distinguishes the two hPR isoforms and does not influence transactivation mediated by AF1 or AF3, but subserves other functions.

The steroid/thyroid receptor family of proteins are ligand-activated transcription factors. Like many other transcription factors, steroid receptors are phosphorylated at sites and for functions that are under intensive study (1–4). The phosphorylation sites of steroid receptors, including chicken and human progesterone receptors (PR),[1] generally (5–10) but not always (11–14) map to serine residues in the N terminus upstream of the DNA binding domain (DBD). One site in cPR maps to the hinge region immediately downstream of the DBD.

Four phosphoserines have been sequenced in cPR; all in Ser-Pro proline-directed kinase consensus sites. Of 5 confirmed sites in hPR, only 1, Ser^{345}, shares homology with a known cPR site (Ser^{260}). Three of the sequenced hPR sites are in BUS, the 164-amino acid B-upstream segment unique to the B-isoform (15, 16). These are Ser^{81}, a Ser-*X*-*X*-Glu casein kinase II (CKII) consensus site, and Ser^{102} and Ser^{162}, both Ser-Pro proline-directed kinase motifs. The 2 other confirmed hPR sites, Ser^{294} and Ser^{345} in the N terminus, are also Ser-Pro suggesting that kinases involved are highly conserved (17–19).

Serine/threonine kinases, including cAMP-dependent protein kinase, mitogen-activated protein kinase, a polypeptide-dependent kinase, CKII, cyclin-dependent kinase (Cdk)2, and double-stranded DNA-dependent kinase, all phosphorylate purified cPR or hPR *in vitro* (15, 16, 20–22). Few sites have been sequenced, however, with the exception of Ser^{81} of hPR which is correctly phosphorylated by CKII *in vitro* and Ser^{528} of cPR which is phosphorylated by cAMP-dependent protein kinase *in vitro* and lies in close proximity to, but is not identical with, the authentic *in vivo* phosphorylated hinge region Ser^{530}.

It remains unclear whether studies showing cross-talk between cell surface signaling pathways and nuclear steroid receptors (11, 23–26) are related to PR phosphorylation. *In vivo* treatments that raise cellular cAMP levels increase cPR-mediated transcription in a ligand-independent manner, but have not been shown to increase phosphate incorporation by the receptors (27–29). Transactivation by hPR is also increased by treatments that raise cellular cAMP levels; an effect that requires ligand occupancy (30, 31). However, the robust transcription produced by synergism between cAMP-dependent protein kinase and ligand-occupied hPR is not accompanied by obvious changes in the phosphorylation state of the receptors. Of the 5 sequenced phosphoserines in hPR and 4 in cPR, none have been shown to be phosphorylated by cAMP-dependent protein kinase or protein kinase C.

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[1] The abbreviations used are: PR, progesterone receptor; cPR, chicken PR; hPR, human PR; DBD, DNA binding domain; HBD, hormone binding domain; BUS, B-upstream segment; NLS, nuclear localization signal; CK, casein kinase; ER, estrogen receptor; GR, glucocorticoid receptor; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; MMTV, murine mammary tumor virus; tk, thymidine kinase; HSV, herpes simplex virus; PRE, progesterone response element; ERE, estrogen response element; mAb, monoclonal antibody; TAT, tyrosine aminotransferase; Ad2MLP, adenovirus 2 major late promoter.

There are two phosphorylation states of hPR: a basal state characteristic of unliganded holoreceptors and a ligand-induced state in which phosphate incorporation is severalfold higher than basal. The ligand-induced hyperphosphorylation of hPR is further subdivided into a DNA binding-independent stage and a DNA binding-dependent stage (32, 33). However, the function of phosphorylation remains unknown. It has been speculated to play a role in regulation of transcription and, indeed, for human estrogen receptors (hER) and mouse glucocorticoid receptors (mGR), modest reductions in transcriptional activity have been observed using site-directed mutants. Bai *et al.* (37) have reported that phosphorylation of Ser^{530} in the hinge region of cPR increases their transcriptional activity; an effect observed only at low ligand concentrations. On the other hand, mutation of all 5 putative phosphoserines in *tau1* of hGR has no effect on transcription (34). Of course, a role for phosphorylation in functions other than transcription is also possible, as, for example, in ligand-induced dimerization or DNA binding (35, 36).

Analysis of phosphorylation function in hPR is complicated by the existence of two isoforms: B-receptors which contain BUS at their N termini and A-receptors which lack it. B- and A-receptors have important functional differences in response to agonists (38–40) and differ extensively when occupied by antagonists (31, 41, 42). These differences appear to reside in BUS, which contains a strong autonomous activation function (AF3) and is heavily phosphorylated (6, 15, 40). No phosphoserines have yet been localized within AF1, which lies in the N terminus just upstream of the DBD. Two phosphoserines map to the region between the A-receptor translation start site and AF1 which includes a region that possesses a transcriptional inhibitory function in the context of A-receptors.[2]

We have undertaken an extensive series of studies to test the role of hPR phosphorylation on DNA binding and transcriptional activity and constructed two series of serine to alanine substitution mutants. The M-series mutants (Fig. 1) involve 10 clusters of serine residues located in the N-terminal arm or hinge region common to both isoforms, cloned into the background of either A- or B-receptors, and include all Ser-Pro and potential CKII motifs in or around AF1. The B-series mutants (Fig. 1) involve 6 serine clusters located in BUS, cloned into BUS-DBD-NLS (40) and into full-length B-receptors, and include all Ser-Pro motifs that might influence AF3.

Using the M-series mutants in the background of full-length B- or A-receptors, we observe no effects on DNA binding with any of the mutants and modest effects on transactivation, dependent on cell and promoter context with 3 out of 10 mutants. Using the B-series mutants, we find that completely dephosphorylated BUS-DBD-NLS constructs retain the strong AF3 transactivating capacity of their wild-type counterparts. Additionally, the unique properties of RU486-occupied full-length B-receptors are retained despite complete BUS dephosphorylation. We conclude that phosphorylation has subtle overall effects on hPR transcription and that neither the activation function of AF3 in BUS, nor of AF1 in the N terminus, is controlled by its phosphorylation state.

MATERIALS AND METHODS

Plasmid Constructions—Complementary DNAs, hPR2 and hPR1, encoding A- and B-receptors, respectively, cloned into the pSG5 expression vector (44) were gifts from P. Chambon (Strasbourg, France). BUS-DBD-NLS cloned into pSG5 was described in Sartorius *et al.* (40). M- and B-series site-specific serine to alanine substitution mutants were made either by oligonucleotide-directed mutagenesis employing a single-stranded template DNA (45) or by polymerase chain reaction (PCR) using overlapping primer products to generate a heteroduplex with the mutant residues placed within a DNA fragment containing convenient restriction sites at the 5′ and 3′ ends (46). For screening purposes, new restriction sites were introduced within or adjacent to the nucleotide sequence associated with the serine to alanine mutation. Individual mutants, particularly those within the B-series BUS-DBD-NLS, were grouped to form combination mutants. The B_{12} mutant was constructed by PCR amplification of a fragment in the B_1 mutant containing *AvrII* and *Sac*I restriction sites at the 5′ and 3′ ends, respectively. This fragment was then subcloned into the large *Avr*II/*Sac*I vector-containing fragment of the B_2 mutant plasmid. Ligation at the *Sac*I site recreated the B_2 mutant resulting in the B_{12} combination mutant. The B_{123} combination mutant was constructed by PCR amplification of an *Avr*II/*Pst*I fragment in the B_{12} mutant plasmid. This fragment was then subcloned into the *Avr*II/*Pst*I-digested B_3 mutant plasmid. The B_{1234} combination mutant was constructed by PCR amplification of a *Pst*I/*Bgl*II fragment from the B_4 mutant plasmid and was subcloned into the *Pst*I/*Bgl*II-digested B_{123} mutant plasmid. The B_{12345} combination mutant was constructed by PCR amplification of a *Pst*I/*Bgl*II fragment from a B_{45} combination mutant plasmid, which was subcloned into the B_{123} combination mutant plasmid. The B_{45} combination mutant plasmid was constructed by digesting the B_4 and B_5 plasmids with *Bst*EII/*Rsr*II and subcloning the small fragment from the B_5 plasmid into the large fragment from the B_4 plasmid. The CK mutant was constructed in both the wild-type BUS-DBD-NLS construct and the BUS_{12345}-DBD-NLS combination mutant plasmids. All BUS individual and combination mutants were inserted into the hPR1 plasmid encoding full-length B-receptors by subcloning either an *Eco*RI/*Bst*EII or *Eco*RI/*Rsr*II fragment from the mutant BUS-DBD-NLS plasmid into the hPR1 wild-type plasmid. All mutants were verified by dideoxynucleotide sequencing. All mutant plasmids were transfected into COS-1 or HeLa cells, and the molecular size and structure of the expressed proteins were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting as described previously (32).

Cell Transfections—Transient transfections into PR-negative COS-1 monkey kidney epithelial, HeLa human cervicocarcinoma, and $T47D_D$ human breast cancer cells were performed by calcium phosphate precipitation as described previously (31). Receptors included wild-type and mutant hPR expression plasmids and the human estrogen receptor (hER) expression vector HEGO (47) (a gift of P. Chambon). Reporter plasmids PRE-tk_{HSV}, PRE_2-$TATA_{Ad2MLP}$, and MMTV-CAT were gifts of P. Chambon. PRE_2-$TATA_{tk}$-CAT was constructed as described previously (31), and the two PREs were replaced by two EREs derived from the vitellogenin promoter to generate ERE_2-$TATA_{tk}$-CAT. β-Galactosidase expression plasmids, pCH110 (Pharmacia Biotech Inc.) or CMV-β-gal (Clontech, Palo Alto, CA), were used to correct for transfection efficiency, and the Bluescribe plasmid (Stratagene) was used as a carrier. Wild-type and mutant expression plasmids were also transiently transfected into HeLa cells containing the stably integrated MMTV-CAT promoter-reporter introduced into these PR-negative cells as described previously (48).

[^{32}P]Orthophosphate Labeling, Immunoprecipitation, and Immunoblotting—COS-1 cells transiently transfected with full-length hPR1, hPR2, or their respective mutants, were treated with R5020 or alcohol vehicle 10 min prior to the addition of [^{32}P]orthophosphate (0.15 mCi/ml of medium) as described previously (6). Cells were harvested 4 to 17 h after incubation with [^{32}P]orthophosphate, homogenized in buffer containing 0.6 M KCl, desalted over Sephadex G-25, and immunoprecipitated with B-30 and/or AB52 monoclonal antibodies. Immunoprecipitated receptors were then subjected to SDS-PAGE, transferred to nitrocellulose, immunoblotted with mAb B-30 and/or AB-52, and the specific bands were visualized on x-ray film by enhanced chemiluminescence (ECL, Amersham), as described previously (48). The sheets were air-dried, the chemiluminescence was allowed to decay over 24 h, and the ^{32}P radioactivity present in receptor-associated bands was visualized by autoradiography of another x-ray film.

Gel Shift Analysis—Gel mobility shift assays were performed as described (31) using whole cell extracts prepared from transfected COS-1 cells. Hormone (0.1 μM R5020) was added 2 h prior to cell harvest. ^{32}P-Labeled oligonucleotide probes were 27 base pairs in length and contained either a palindromic progesterone response element (PRE) from the tyrosine aminotransferase (TAT) promoter, or the distal palindromic PRE of the MMTV long terminal repeat (30, 32).

CAT Assays—Twenty-four h after transfection, the cell medium was changed and the cells were incubated with or without R5020 for an additional 24 h. Cells were then harvested, and lysates were analyzed for chloramphenicol acetyltransferase (CAT) activity by thin layer chro-

[2] A. Rudie Hovland, R. L. Powell, G. S. Takimoto, L. Tung, and K. B. Horwitz, submitted for publication.

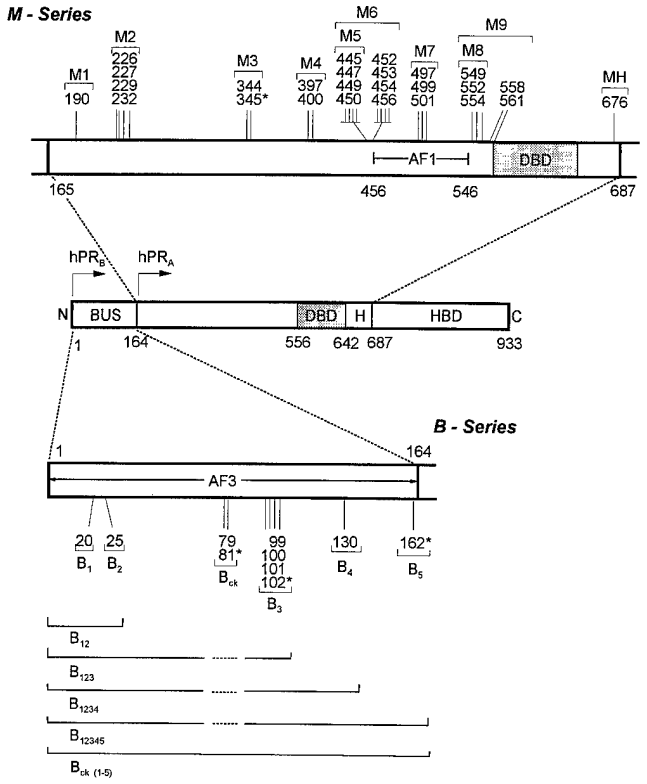

FIG. 1. **Human PR serine to alanine substitution mutants tested in this study.** The *center bar* shows the major structural domains of hPR and their amino acid borders including the B-upstream segment (*BUS*), the translation start site for B-receptors (hPR_B) and A-receptors (hPR_A), the DBD, hinge region (*H*), and HBD. The 10 M-series mutants (*M1* to *M9* and *MH*), shown on *top*, contain clusters of 1–8 serine to alanine mutations, located between the A-receptor start site and the end of the hinge region. Also shown is the position of a 91-amino acid activation domain, AF1. The six B-series mutants (B_1 to B_5 and B_{ck}), shown in the *lower bar*, contain clusters of 1–4 serine to alanine mutations located within BUS. Additional BUS mutants involve two or more serine clusters, as shown. In $B_{CK(1-5)}$, all 10 serine residues are mutated. The * indicates serines known to be phosphorylated *in vivo* (15, 16).

matography (TLC) and quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA), as described previously (40).

RESULTS AND DISCUSSION

Phosphorylation Mutants—The specific amino acids mutated, and their designations are illustrated in Fig. 1. Among the mutated residues are ones that have been identified as phosphoserines in hPR (* in Fig. 1) including Ser^{81} (B_{CK}), Ser^{102} (B_3), Ser^{162} (B_5), and Ser^{345} (M3); ones that have been identified as phosphoserines in cPR and bear homology to sites in hPR including Ser^{345} (M3) and Ser^{676} (MH); and ones that represent consensus phosphorylation sites for CKII and proline-directed kinases and have at least a Ser-Pro motif. In the M-series mutants, 10 clusters of serine residues located downstream of Met^{165} in regions common to both PR isoforms were mutated in expression vectors encoding both isoforms. In the B-series mutants, 6 clusters of serine residues located in BUS were mutated in BUS-DBD-NLS and in the full-length B-receptors. Additional B-series mutants contained two or more of the mutant clusters in various combinations, and, in the CK (1–5) construct, all 6 serine clusters in BUS were mutated simultaneously.

M-Series Mutants: Phosphorylation Sites Common to Both A- and B-Receptors—Initial studies involved a series of 9 different serine to alanine mutant clusters located in the N terminus of A-receptors upstream of the DBD (designated M1 to M9) and 1 located in the hinge region downstream of the DBD (designated MH) (Fig. 1). Mutants M5 to M9 either surround or are located within the AF1 transcription activation domain. The mutant proteins were all well expressed as demonstrated by immunoblotting (not all shown, but see Fig. 3). They were tested for transcriptional activity by transient cotransfection with the minimal PRE_2-$TATA_{Ad2MLP}$ and PRE_2-$TATA_{tk}$ promoters or the complex PRE-tk_{HSV} promoter, using either HeLa or COS cells. Transcription of the CAT gene by a majority of these constructs when occupied by R5020 was no different than transcription by wild-type A-receptor controls (data not shown). Three sets of A-receptor mutants, M1, M9, and MH, described in Fig. 1, have some inhibitory effects on transcription, that are promoter- and cell-specific (Figs. 2, *A* and *B*). In COS cells (Fig. 2*A*), M1 mutants were less active on the simple PRE_2-$TATA_{Ad2MLP}$ promoter than on the complex PRE-tk_{HSV} promoter; M9 and MH were weaker receptors than wild-type A-receptors on both promoters. In HeLa cells (Fig. 2*B*), transcription controlled by M1 and M9 is variable and promoter-dependent, and no clear rules can be deduced. The MH hinge region mutation at Ser^{676} appears to have the most consistent deleterious effect on A-receptor activity. The data in Fig. 2 represent average values for duplicates of 2–8 assays per set and include the range of variability among assays. The overall impression is that these three mutants have a 20–50% transcription inhibitory effect. Mutants M1, M9, and MH were also cloned into the B-receptor expression vector and tested on PRE_2-$TATA_{tk}$ in HeLa cells, with results analogous to those seen with their A-receptor counterparts (data not shown).

We conclude that phosphorylation of Ser^{190}, the M9 cluster, or Ser^{676} has subtle effects on hPR transcription. Bai *et al.* (37) have reported that mutation of Ser^{530} in cPR (which is homologous to mutant MH at Ser^{676} in hPR) reduces receptor-mediated transcription in transient transfections assays, but only at low hormone concentrations. We observe a transcriptional decrement even at saturating hormone concentrations with MH. Since there is no evidence that Ser^{676} is phosphorylated in hPR, it is possible that the decrement in transcriptional activity observed with MH is due to disruption of a function of this domain independent of a phosphorylation event (49). Our results with hPR are analogous to those obtained with hER, in which site-directed N-terminal (AF1) mutants (24, 50) also produced modest cell- and promoter-specific reductions in transcriptional activity in transient transfection assays. Similarly, mutation of all 7 phosphorylated residues in the N terminus of hGR, 6 of which lie within *tau1*, reduced transcription by 30–40% in transient assays (51). However, when this 7-site mGR mutant was expressed at physiological levels, transcription by the mutant was equivalent to the wild-type mGR. These findings illustrate the complexities involved in assessing subtle functional effects using overexpressed mutant receptors in transient transfection assays.

The three M-series mutants of interest were also analyzed for their ability to undergo phosphorylation-dependent structural changes (Fig. 3*A*) and for their ability to bind DNA at a PRE (Fig. 3*B*). For these studies, wild-type A-receptors and all the M-series mutants including the M1, M9, and MH mutants were expressed in COS cells, treated with R5020, or left untreated, and the extracted receptors were analyzed by immunoblotting and gel mobility shift assays. As we have shown previously (17), unactivated wild-type A-receptors immunoblot as singlets (Fig. 3*A*, *solid arrow*), but, after activation by hormone, they migrate as doublets on electrophoretic gels (Fig. 3*A*, *open arrow*) due to a hormone-dependent phosphorylation step. Analogous to wild-type A-receptors, M1, M9, and MH are also singlets in the absence of hormone and are upshifted by hor-

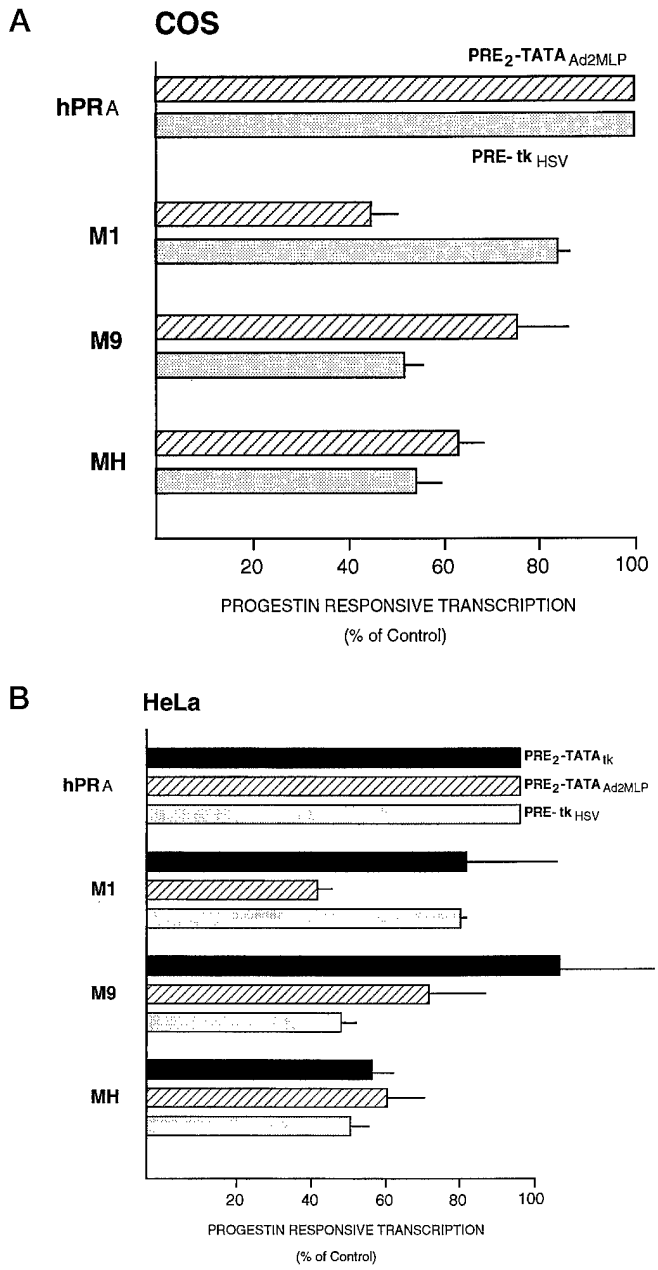

FIG. 2. **Transcriptional activity of M-series mutants according to cell and promoter tested.** COS cells (*A*) or HeLa cells (*B*) were cotransfected with 250 ng of the expression vectors encoding wild-type A-receptors (hPR_A) or the M1, M9, or MH mutants (Fig. 1) and the promoters shown, driving a CAT reporter. Cell extracts from R5020 treated cells, normalized to β-galactosidase activity, were analyzed for CAT activity by TLC, quantified by phosphorimaging, and expressed graphically as a percentage of the acetylated [^{14}C]chloramphenicol levels generated by wild-type A-receptors. *Bars* indicate the average (±S.D.) of 2–8 assays, each performed in duplicate.

mone occupancy. We conclude that the serines mutated in these three constructs are not targets for the hormone-dependent phosphorylation that produces the upshift. Mutation of 6 other serine clusters in the N terminus of A-receptors (see Fig. 1) also had no effect on their immunoblotting pattern (data not shown). On the other hand, the M3 mutant, which includes Ser^{345}, is upshift-deficient (data not shown) consistent with recent reports of Zhang *et al.* (15). Thus, the hormone-dependent upshift appears to be unrelated to transcriptional activity, since a mutant lacking the upshift (M3) is fully active, while mutants with a normal upshift (M1, M9, MH) are transcriptionally deficient.

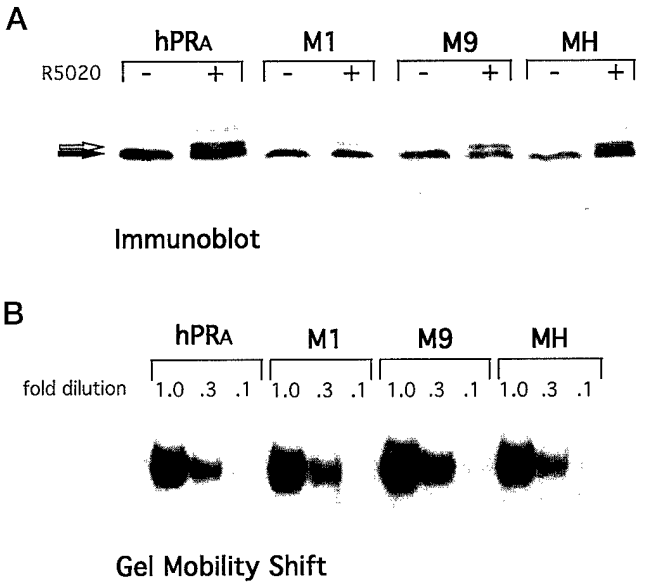

FIG. 3. **Immunoblot and DNA-binding analyses of M-series mutants.** Nuclear extracts were prepared from COS cells transfected with wild-type A-receptors or the three M-series mutants indicated and treated (+ and *B*) or not (−) with R5020. *A*, immunoblot probed with mAb AB-52. The hormone-dependent upshift is indicated by the *open arrow*. *B*, gel mobility shift assay using various nuclear extract concentrations and a constant amount of the [^{32}P]PRE-containing oligonucleotide.

Other recent studies (13, 35, 52) have suggested that phosphorylation of steroid receptors regulates their DNA binding capacity. To test this, wild-type A-receptors and the M1, M9, and MH mutants were expressed in COS cells in the presence or absence of a saturating concentration of R5020. The receptors were extracted and incubated with a 27-base pair ^{32}P-labeled oligonucleotide containing either the distal palindromic PRE of the MMTV long terminal repeat (not shown) or a palindromic PRE from the TAT promoter (Fig. 3*B*). Receptor-DNA complexes at three different extract concentrations were then analyzed by the *in vitro* gel mobility shift assay. Fig. 3*B* shows that there is no remarkable difference in DNA binding affinity between wild-type and mutant A-receptors. Similar conclusions were drawn from a study comparing wild-type B-receptors and their corresponding M1, M9, and MH mutants (data not shown). It is unlikely, therefore, that altered DNA binding activity or differences in protein expression levels account for the reductions in transcription seen with the M1, M9, and MH mutants, since the PRE used in the gel mobility shift assay was also inserted into all the reporter plasmids, and comparable levels of wild-type and mutant receptors were expressed from transiently transfected COS cells (see Fig. 3*A*).

Phosphorylation Sites Unique to B-Receptors: the B-Series Mutants—We have previously shown that PR B- and A-receptors have important functional differences due to an AF3 present in BUS (40). BUS is also highly phosphorylated (6). The triplet immunoblotting banding pattern of full-length 120-kDa B-receptors, which is due to phosphorylation, is entirely reproduced by the 20-kDa BUS fragment (see Fig. 4*B*). Because of its strong transactivating capacity and intensive phosphorylation, BUS-DBD-NLS is an ideal receptor fragment with which to test the functions of phosphorylation. We therefore constructed a set of BUS phosphorylation mutants in which 6 clusters of serine residues were individually or collectively mutated. Five of these clusters (B_1 to B_5) contain Ser-Pro phosphorylation motifs; the sixth (B_{CK}) has a CKII phosphorylation motif. B_1 is mutated at Ser^{20}, B_2 at Ser^{25}, B_3 at $Ser^{99,100,101,102}$, B_4 at Ser^{131}, B_5 at Ser^{162}, and B_{CK} at $Ser^{79,81}$ (Fig. 1). Three serines

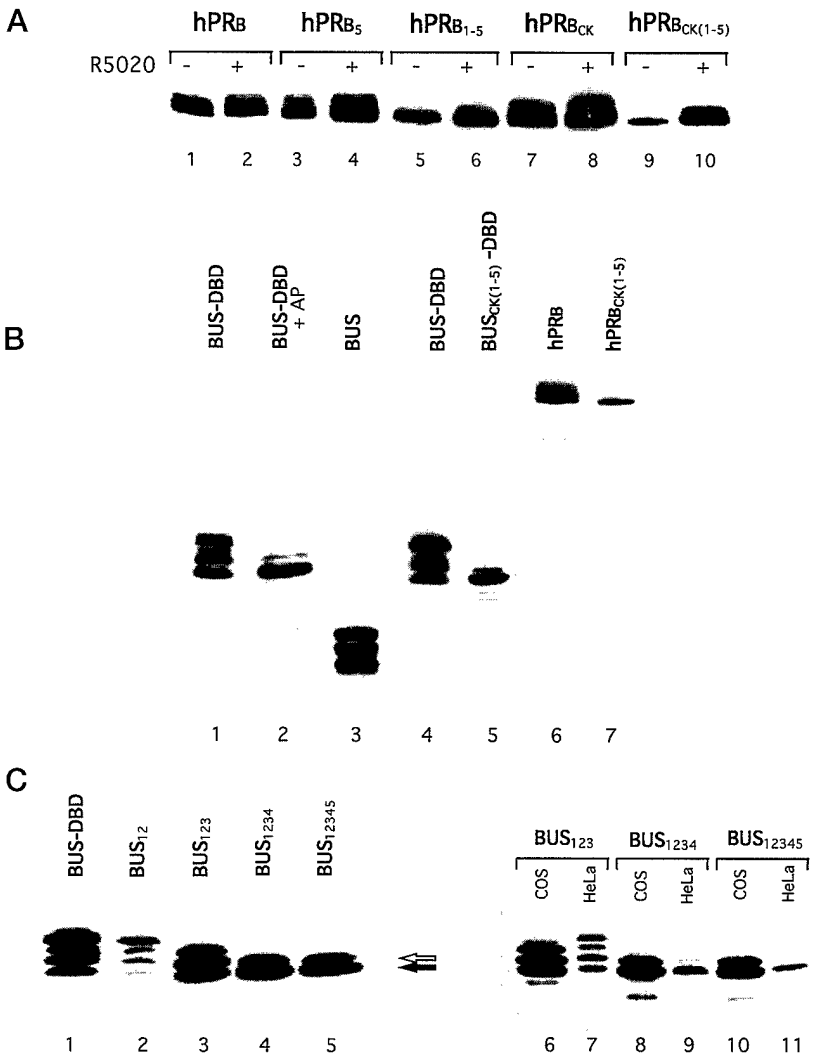

FIG. 4. **Immunoblot analyses of B-series phosphorylation-deficient mutants.** Nuclear extracts from R5020-treated (+) or untreated (−) HeLa or COS cells transiently expressing wild-type B-receptors or BUS-DBD-NLS or the corresponding phosphorylation-deficient mutants shown. Extracts were separated on SDS-PAGE and immunoblotted with the B-receptor-specific mAb B-30. *A*, full-length B-receptors and selected phosphorylation mutants expressed in COS cells. *B*, full-length B-receptors and BUS-DBD-NLS constructs expressed in COS cells. Wild-type BUS-DBD-NLS (*lane 1*); alkaline phosphatase (*AP*)-treated BUS-DBD-NLS (*lane 2*); removal of DBD (*lane 3*). Wild-type BUS-DBD-NLS and hPR_B constructs (*lanes 4* and *6*) and corresponding 6-site BUS mutants (*lanes 5* and *7*). *C, left panel*, wild-type (*lane 1*) or BUS-DBD-NLS constructs carrying 2 (*lane 2*) to 5 (*lane 5*) serine cluster mutations isolated from COS cells. *Open arrow* indicates the "upshifted" band. *Right panel*, comparison of BUS-DBD-NLS mutants expressed in HeLa cells and COS cells carrying 3 (*lanes 6* and *7*), 4 (*lanes 8* and *9*), or 5 (*lanes 10* and *11*) serine cluster mutations.

in these constructs, Ser^{102} in B_3, Ser^{162} in B_5, and Ser^{81} in B_{CK} have been sequenced (* in Fig. 1) and are known to be phosphoserines (15, 16). Additionally, B_1 was combined with B_2 to yield B_{12}, and, similarly, B_{123}, B_{1234}, and B_{12345} were constructed. Finally, all 6 clusters were simultaneously mutated in a construct called $B_{CK(1-5)}$ (Fig. 1). The BUS mutants were inserted into BUS-DBD-NLS and into full-length B-receptors.

Fig. 4 shows immunoblots that demonstrate structural features of some of these mutants, which are well expressed suggesting that their stability is not altered by the BUS mutations. Full-length B-receptors transiently expressed in COS cells resolve as triplets on electrophoretic gels (Fig. 4*A*) and resemble natural B-receptors isolated from breast cancer cells. Addition of hormone has minor effects on the banding pattern (compare *lanes 1* and *2*) since the hormone-dependent M_r upshift observed in other cells is less prominent in COS cells (see Fig. 4*C*). Mutation of any one Ser-Pro motif, as in the B_5 (Fig. 4*A*, *lanes 3* and *4*) or the B_{CK} (*lanes 7* and *8*) mutants does not alter the immunoblotting pattern. These two represent serine residues that are known to be phosphorylated *in vivo*. However, the multiple banding pattern is reduced to a singlet if 5 (*lane 5*) or all 6 (*lane 9*) serine clusters are mutated. Nevertheless, at least 1 hormone-dependent phosphorylation site is retained in these constructs (*lanes 6* and *10*), since the M_r upshift occurs after R5020 treatment (compare *lanes 9* and *10*, for example) confirming that this site(s) lies downstream of BUS in the region common to both PR isoforms (15).

BUS-DBD-NLS also immunoblots as a triplet (Fig. 4*B*, *lane 1*) due to phosphorylation of sites located in BUS (*lane 3*) as we have shown previously (40). The complexity of this pattern, coupled with high performance liquid chromatography analysis of tryptic phosphopeptides (6, 16) suggest that it is due to phosphorylation of multiple serine residues. If BUS-DBD-NLS is treated with calf intestinal alkaline phosphatase, the higher M_r hyperphosphorylated bands can be reduced or eliminated (Fig. 4*B*, *lane 2*). This is also demonstrated using the $BUS_{CK(1-5)}$-DBD-NLS mutant in which the upper two bands are extensively reduced (*lane 5*), compared to the wild-type construct which immunoblots as three or more bands (Fig. 4*B*, *lane 4*). Complete reduction of the triplet to a singlet is seen in full-length B-receptors in which all 6 serine phosphorylation motifs present in BUS ($B_{CK(1-5)}$) have been mutated (Fig. 5*B*; compare *lanes 6* and *7*). We tentatively conclude that CK(1–5) mutants are entirely dephosphorylated at the sites unique to B-receptors.

Immunoblot analyses of wild-type BUS-DBD-NLS and its mutants carrying single or intermediate numbers of serine substitutions are also informative about generation of the triplet structure (Fig. 4*C*). Regardless of the site involved, mutation of any 1 of the 6 serine clusters, produces no discernible change in the immunoblot banding pattern (data not shown, but see Fig. 4*A*). As shown in COS cells, even mutation of 2 of the 6 clusters (B_{12}) produces no diminution in the number of blotted bands (Fig. 4*C*, *lane 2*). Only after 3 (B_{123}) or more

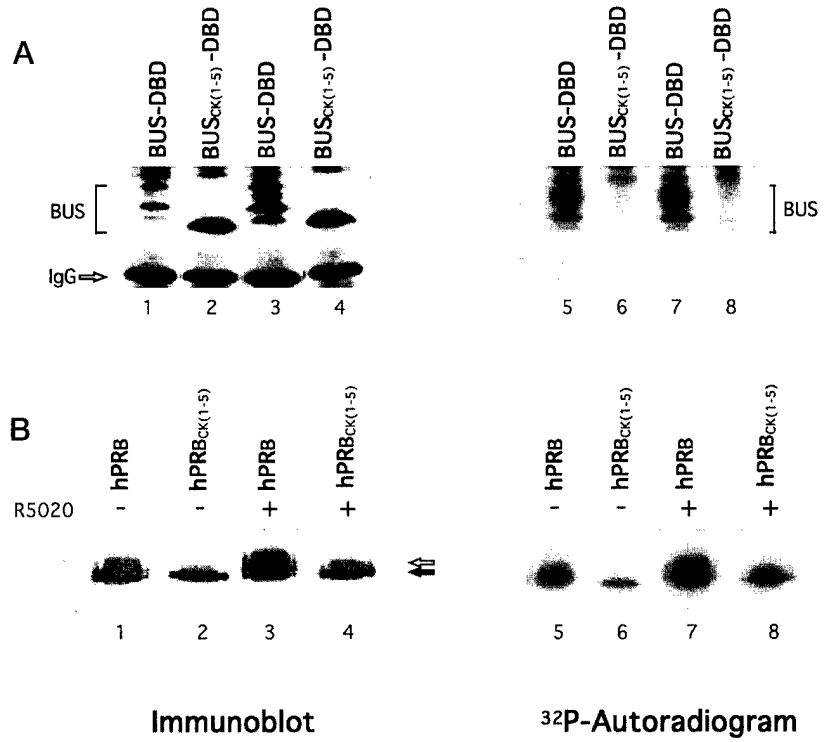

FIG. 5. **Analysis of the phosphorylation state of B-series mutants based on [^{32}P]orthophosphate incorporation in intact cells.** COS cells expressing the indicated BUS-DBD-NLS or full-length hPR_B constructs were incubated with [^{32}P]orthophosphate for 4 h, nuclear extracts were immunoprecipitated with mAb B-30 alone or together with AB-52, separated by SDS-PAGE, transferred to nitrocellulose, and the sheet was visualized by enhanced chemiluminescence (*left panels*), then dried overnight and exposed to another x-ray film to generate the autoradiogram (*right panels*). *A*, comparison of the protein structure (*left panel*) and [^{32}P]orthophosphate incorporation (*right panel*) of wild-type BUS-DBD-NLS or the corresponding 6-site CK(1–5) BUS mutant. *B*, comparison of the protein structure (*left panel*) and [^{32}P]orthophosphate incorporation (*right panel*) of full-length B-receptors or the corresponding 6-site CK(1–5) BUS mutant.

(B_{1234} and B_{12345}) clusters are mutated does the pattern begin to converge to a single band (*lanes 3–5*). However, even mutation of 5 of the 6 sites, as for example in mutant B_{12345} (Fig. 4*C*, *lane 5*), still yields a weak doublet (*open arrow*). These data suggest that there is considerable intramolecular heterogeneity among the sites that are phosphorylated *in vivo* and that phosphorylation at several alternative combinations of sites can produce the complex triplet banding pattern, as has been described for vitamin D receptors (53).

Also shown in Fig. 4*C*, *lanes 6–11*, is a comparison of the immunoblotting pattern of three BUS-DBD-NLS mutants when they are expressed in HeLa cells or COS cells. It demonstrates subtle differences in the phosphorylation pattern produced by the two cell lines that may reflect differences in cellular kinases, differences in the residues that are their targets, or possibly differences in protein expression levels which are usually lower in HeLa cells.

The studies shown in Fig. 5 demonstrate directly that the 6-site BUS mutant, CK(1–5), is completely dephosphorylated. COS cells transiently expressing wild-type BUS-DBD-NLS or the $BUS_{CK(1-5)}$-DBD-NLS mutant were incubated with [^{32}P]orthophosphate. The labeled receptors were then extracted, immunoprecipitated, resolved by gel electrophoresis, transferred to nitrocellulose, and analyzed by both ^{32}P autoradiography (*right panels*) and by immunoblotting with mAb B-30 (*left panels*). Hormone treatment was unnecessary, since the constructs lack an HBD and are constitutive transactivators (40). The immunoblot in Fig. 5*A* shows the characteristic multiple banding pattern of wild-type BUS-DBD-NLS (*lanes 1* and *3*) and its reduction to a singlet band in the $BUS_{CK(1-5)}$ mutant (*lanes 2* and *4*). The parallel autoradiogram shows that all the protein bands are phosphorylated in wild-type BUS-DBD-NLS (*lanes 5* and *7*), but that in $BUS_{CK(1-5)}$-DBD-NLS, even the heavy singlet protein band (*lanes 2* and *4*) is dephosphorylated (*lanes 6* and *8*). This confirms that in the 6-site mutant no residues remain that are substrates for endogenous serine kinases, and that no other amino acid residues become alternatively phosphorylated when the fully mutated $BUS_{CK(1-5)}$-DBD-NLS construct is expressed.

Fig. 5*B* is a similar analysis of COS cells transiently expressing full-length B-receptors that contain either wild-type BUS or the 6-site $B_{CK(1-5)}$ mutant BUS. Because these receptors have an HBD, the cells were either untreated (−) or treated with R5020 (+) before the receptors were extracted. In the absence of hormone, the characteristic triplet immunoblot banding pattern is observed with wild-type B-receptors (*lane 1*) and reduced to a singlet in the mutant (*lane 2*). After hormone occupancy, a slightly shifted banding pattern is observed in the immunoblot of wild-type B-receptors (*lane 3*), characteristic of COS cells (see Fig. 4). The $B_{CK(1-5)}$ mutant (*lane 4*) also shifts from a singlet (*lane 2*, *solid arrow*) to a doublet (*lane 4*, *open arrow*) following hormone occupancy, due to phosphorylation of 1 or more sites downstream of BUS. The parallel ^{32}P autoradiogram shows that the basal phosphorylation of wild-type B-receptors (*lane 5*) is augmented by hormone treatment (*lane 7*), as we have previously reported (6). That this hormone-dependent hyperphosphorylation is not due to sites in BUS is shown by the $B_{CK(1-5)}$ mutant in which a 4-fold increase in [^{32}P]orthophosphate incorporation is observed following hormone treatment (*lane 8*) compared to the untreated control (*lane 6*).

BUS-DBD-NLS serves as a powerful tool to study functions of phosphorylation because of its strong constitutive transactivating capacity. We have therefore extensively analyzed the DNA binding properties and transcription regulatory properties of constructs containing either a wild-type or a phosphorylation-deficient BUS. We have previously reported (40) that wild-type BUS-DBD-NLS binds strongly to DNA at a PRE if a nuclear accessory protein, or the bivalent mAb B-30, is included in the DNA-bound complex. We find an identical DNA binding pattern with the $B_{CK(1-5)}$ mutant (data not shown). Thus, elimination of BUS phosphorylation does not influence the DNA binding capacity of the BUS-DBD-NLS construct or its ability to interact with the nuclear accessory protein.

We have also carried out extensive transcription analyses comparing fully phosphorylated wild-type BUS-DBD-NLS and full-length B-receptors, with their counterparts containing single-site and multi-site BUS phosphorylation-deficient mutants.

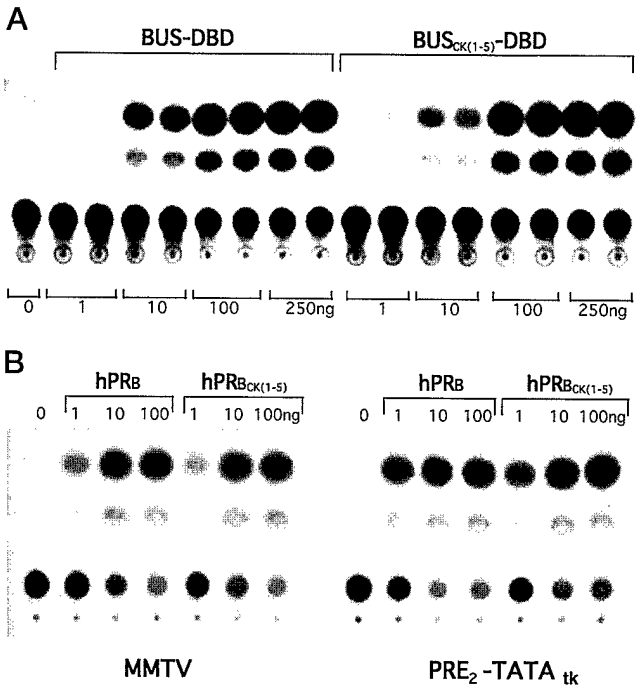

FIG. 6. **Transcriptional activity of wild-type full-length B-receptors or BUS-DBD-NLS and their corresponding 6-site phosphorylation-deficient mutants.** HeLa cells were transiently transfected with 2 μg of PRE$_2$-TATA$_{tk}$-CAT or MMTV-CAT reporters, 2 μg of β-galactosidase expression vector, and 1–250 ng of the wild-type or mutant receptor expression vectors, as shown. Cell lysates were normalized to β-galactosidase activity, and CAT expression was analyzed by TLC and quantified by phosphorimaging. *A*, BUS-DBD-NLS constructs and PRE$_2$-TATA$_{tk}$-CAT. *B*, full-length B-receptor constructs and MMTV-CAT or PRE$_2$-TATA$_{tk}$-CAT.

The constructs all have remarkably similar transcriptional activities. An example of such a study, comparing transcription from the PRE$_2$-TATA$_{tk}$-CAT reporter cotransfected into HeLa cells together with increasing concentrations of expression vectors encoding wild-type BUS-DBD-NLS, or the 6-site mutant BUS$_{CK(1-5)}$-DBD-NLS, is shown in Fig. 6. There is a constitutive, dose-dependent increase in transcription by wild-type BUS-DBD-NLS, which, at its peak, is equivalent to transcription by full-length B-receptors (40). Surprisingly, transcription by the completely dephosphorylated BUS$_{CK(1-5)}$-DBD-NLS mutant is essentially identical with that of its fully phosphorylated counterpart (Fig. 6*A*). Minor effects of dephosphorylation are observed at low DNA input concentrations. In Fig. 6*A*, for example, transcription following transfection by 10 ng of the cDNA encoding wild-type BUS-DBD-NLS is 23% of the maximum seen at 250 ng, while at 10 ng of the cDNA encoding BUS$_{CK(1-5)}$-DBD-NLS, transcription is 5% of maximum. However, at higher cDNA concentrations, both constructs produce equivalent amounts of CAT activity, and we conclude that the phosphorylation state of BUS has little or no influence over transcription by AF3 in the context of BUS-DBD-NLS.

Fig. 6*B* shows CAT transcription in R5020-treated HeLa cells driven from the MMTV or PRE$_2$-TATA$_{tk}$ promoters, under the control of full-length hPR$_B$ containing either wild-type BUS or completely dephosphorylated BUS$_{CK(1-5)}$. Clearly, there are no remarkable differences between wild-type hPR$_B$ and ones carrying dephosphorylated BUS, regardless of the receptor concentration introduced into the cells. We conclude that the phosphorylation state of BUS has little or no influence over AF3 activity in the context of full-length B-receptors.

Similar conclusions were reached using BUS-DBD-NLS constructs and MMTV-CAT when the reporter was stably transfected into HeLa cells (data not shown). HeLa cells with a stably replicating MMTV-CAT template were constructed and analyzed because of the possibility that PR vary in their ability to activate chromosomal *versus* transiently introduced promoters (54). We postulated that the state of PR phosphorylation might explain these differences, but conclude that they do not.

Phosphorylation and Antagonist Actions in B-Receptors: the B-Series Mutants—There are important quantitative differences between the two PR isoforms when they are occupied by agonists (38–40). However, when the two isoforms are occupied by antagonists, differences between them are profound (31, 41, 42). For example, through cross-talk with the cAMP signaling pathway, B-receptors occupied by the antiprogestin RU486 become strong transcriptional activators under conditions in which RU486-occupied A-receptors inhibit transcription. Since B- and A-receptors differ only by the presence or absence of BUS, we asked whether their phosphorylation state influences the unique properties of B-receptors. In Fig. 7, full-length wild-type B-receptors, or their 6-site B$_{CK(1-5)}$ counterparts, were transiently transfected into PR-negative T47D$_D$ breast cancer cells (55) together with an MMTV-CAT reporter, and the cells were untreated or treated with R5020 or RU486, with or without 8-Br-cAMP. *Lanes 1–5* show that T47D$_D$ cells transiently transfected only with MMTV-CAT are unresponsive to any treatments because they lack PR. If wild-type B-receptors are introduced into the cells together with MMTV-CAT (*lanes 6–13*), there is no CAT synthesis in the absence of hormone (*lane 12*), but CAT levels are high following R5020 treatment (*lane 13*). RU486 (*lanes 6* and *7*) or 8-Br-cAMP (*lanes 10* and *11*) alone is unable to activate transcription, but when the two are combined (*lanes 8* and *9*), strong CAT activity is observed. Since this unusual synergism between 8-Br-cAMP and RU486 occurs only with B-receptors, we asked whether it is dependent on the phosphorylation state of BUS. The B$_{CK(1-5)}$ mutant (*lanes 14–21*) strongly stimulates transcription when occupied by R5020 (compare *lanes 20* and *21*); RU486 (*lanes 14* and *15*) and 8-Br-cAMP (*lanes 18* and *19*) alone are inactive; and the combination of RU486 plus 8-Br-cAMP (*lanes 16* and *17*) is strongly active. We conclude that this unique agonist-like effect of RU486-occupied B-receptors in synergy with cAMP is not dependent on the phosphorylation state of BUS, and, that despite its complete dephosphorylation, BUS can still support this property.

Another interesting functional difference between the two hPR isoforms is that, when occupied by RU486, A-receptors but not B-receptors inhibit transcription of an estrogen response element (ERE) regulated promoter activated by estradiol-occupied hER. BUS blocks this repressor effect of A-receptors.[2] We asked, in the study shown in Fig. 8, whether this property would be lost by a dephosphorylated BUS. For this, HeLa cells were transiently transfected with the wild-type ER expression vector HEGO (47) either alone or together with expression vectors for wild-type B- or A-receptors or the B$_{CK(1-5)}$ receptor mutant. Cells were treated or not with 17β-estradiol (*E*) and RU486 (*RU*), and transcription was measured from the ERE$_2$-TATA$_{tk}$-CAT reporter. This promoter lacks a PRE and cannot be influenced by PR directly. As shown in Fig. 8, the ERE$_2$-TATA$_{tk}$-CAT reporter is not transcribed by ER in the absence of estradiol (*lane 1*) but is strongly transcribed in its presence (*lane 2*). As expected, in the absence of PR, RU486 (*lane 3*) has no influence on this ER-activated, ERE-regulated promoter. When wild-type B-receptor expression vectors are co-transfected with ER (*lanes 4* and *5*), RU486 still has no effect, but with co-transfected wild-type A-receptors (*lanes 6* and *7*), ER-driven transcription is reduced by more than 90%. Note that this inhibitory effect of A-receptors is DNA binding independent, since the promoter lacks a PRE. Despite mutation of all

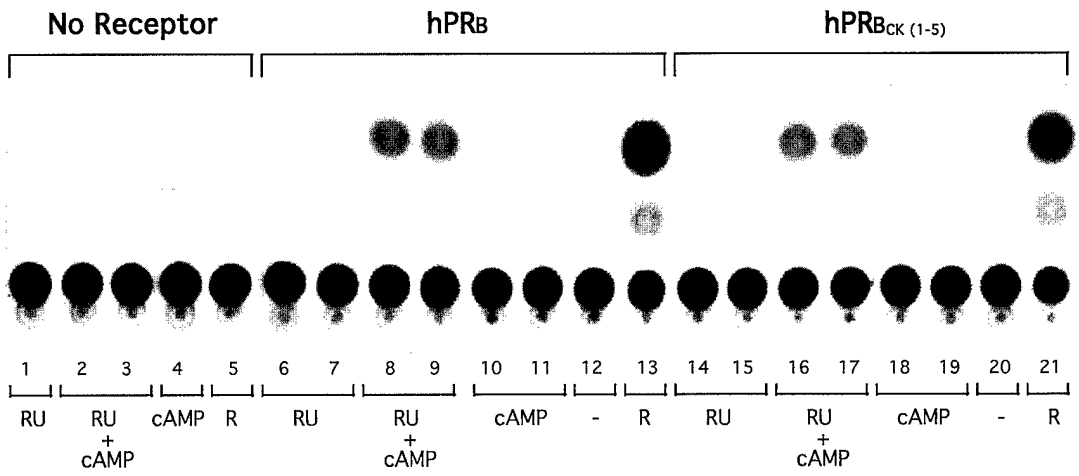

FIG. 7. **Antagonist-occupied B-receptors that are phosphorylation-deficient become transactivators when cAMP levels are raised.** $T47D_D$ cells were transiently transfected with 1 μg of the MMTV-CAT reporter and 1 μg of the pSG5 expression vector alone (*lanes 1–5*) or the vector encoding wild-type B-receptors (*lanes 6–13*), or the 6-site BUS mutant (*lanes 14–21*). Twenty-four hours after transfection, cells were either untreated (−) or treated with 1 mM 8-Br-cAMP (*cAMP*), 50 nM R5020 (*R*), 100 nM RU486 (*RU*), or the indicated combinations for 24 h. Cell lysates were normalized to β-galactosidase activity, and CAT assays were performed by TLC and quantified by phosphorimaging.

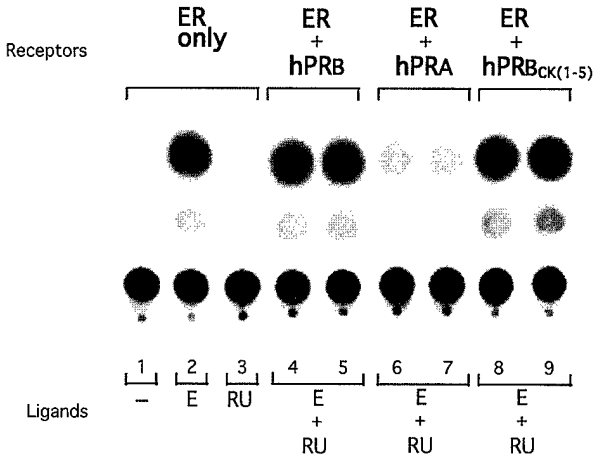

FIG. 8. **Full-length B-receptors carrying a completely dephosphorylated BUS do not acquire the inhibitory phenotype of A-receptors.** HeLa cells were transiently co-transfected with 2 μg of ERE_2-$TATA_{th}$-CAT and 5 ng of the ER expression vector HEGO, with or without 250 ng of expression vectors for full-length A- or B-receptors, or the 6-site BUS mutant B-receptors. Cells were untreated (−) or treated with 10 nM 17β-estradiol (*E*) and/or 100 nM RU486 (*RU*) as shown. Cell extracts normalized to β-galactosidase activity were analyzed for CAT activity as described.

BUS phosphorylation sites in $B_{CK(1-5)}$ (*lanes 8* and *9*), this dominant repressor activity of A-receptors cannot be reconstituted in B-receptors. We conclude again, based on a different experimental model, that factors other than the phosphorylation state of BUS control the unique transcriptional properties of full-length B-receptors.

Summary—In summary, we asked whether phosphorylation of hPR regulates their DNA binding and transcriptional properties. We mutated a number of putative or known phosphorylation sites in the N-terminal region (the M-series mutants) common to the A- and B-isoforms. Many of these sites are either within or bordering AF1, but most mutations had no appreciable effects on transcription by either isoform. Two mutants (M1, M9) in the N terminus and one in the hinge region (MH) produced modest decrements in transcription comparable in magnitude to those seen with mutant hER and mGR (24, 50, 51). If these effects are authentic, it would suggest that receptor phosphorylation does not function as an on/off switch, but rather as a fine-tuning mechanism. On the other hand, if phosphorylation of steroid receptors does not affect receptor-activated transcription as has been shown for hGR and rabbit PR (34, 43), it suggests that receptor processes not directly linked to transcription should be explored.

Similarly, through a combination of site-directed serine to alanine mutations in the BUS region of B-receptors (the B-series mutants), we were able to generate a phosphorylation-deficient AF3 activation domain, which in wild-type B-receptors is highly phosphorylated at multiple serine residues. We studied the autonomous activity of AF3 in BUS-DBD-NLS and its cooperativity with AF1 and AF2 in the context of full-length B-receptors in transfection assays utilizing (*a*) cultured cells derived from different tissues, (*b*) simple and complex promoters, (*c*) different levels of protein expression, and (*d*) templates that are transiently or stably introduced and presumably contain a poorly or a more regularly organized nucleosome structure. Under these extremes of assay conditions, the autonomous transcription efficiency of AF3, as well as its ability to additively or synergistically complement the activities of AF1 and AF2 in the full-length receptors, was essentially unaffected by the mutations that dephosphorylate BUS. Even when we examined functional responses that are specific for B-receptors, such as the agonist activity of antagonist-bound B-receptors in the presence of cAMP, or the inability of B-receptors to be dominant-negative inhibitors of ER, we again found that receptors which were fully phosphorylated or dephosphorylated in BUS acted identically. These B-receptor-specific responses have an absolute requirement for BUS and presumably are mediated by conformational changes in BUS that lead to altered intra- or intermolecular interactions. It is therefore surprising that the intense phosphorylation seen on the BUS fragment is not involved in these activities, but we can come to no other conclusion.

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A CELL CYCLE RESTRICTION POINT CONTROLLED BY PROGESTERONE SENSITIZES BREAST CANCER CELLS TO CROSS-TALK WITH EPIDERMAL GROWTH FACTOR

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Summary

Depending on the tissue, progesterone is classified as a proliferative or a differentiative hormone. To explain this paradox, and to simplify analysis of the effects of progesterone, we used a breast cancer cell line (T47D-YB) that constitutively expresses the B-isoform of progesterone receptors (PR). These cells are resistant to the proliferative effects of epidermal growth factor (EGF). Progesterone treatment accelerates T47D-YB cells through the first mitotic cell cycle, but arrests them in late G1 of the second cycle. Additional progesterone cannot restart cell proliferation despite adequate levels of transcriptionally competent PR. The acquired progesterone resistance is accompanied by decreased levels of cyclins D1, D3 and E, disappearance of cyclins A and B, and induction of the cdk inhibitors p21 and $p27^{Kip1}$. The retinoblastoma protein product is hypophosphorylated and extensively down-regulated. Upregulation of p21 is prolonged and $p27^{Kip}$ levels rise even higher, following progesterone retreatment, intensifying the resistance in an autoinhibitory loop. However, despite progesterone resistance, the cell cycling machinery is poised to restart, since progesterone pretreatment reverses the EGF-resistance and transiently sensitizes the cells to the proliferative effects of EGF, during a narrow time interval in which p21 inhibitory levels are depressed. We propose that progesterone is neither inherently proliferative nor antiproliferative, but that it regulates a cell-cycle restriction point upon which other, tissue-specific factors, influence the fate of the cell.

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Introduction

Progesterone is involved in the development, growth and differentiation of the breast and breast cancers (Clarke and Sutherland, 1990; Horwitz, 1992), and presence of progesterone receptors (PR) identifies tumors likely to be hormone-dependent (Horwitz et al., 1975) and patients likely to have a favorable disease prognosis (Clark et al., 1983). Mice lacking PR exhibit incomplete mammary gland ductal branching and failure of lobulo-alveolar development (Lydon et al., 1995). This phenotype is strikingly similar to that of mice lacking cyclin D1 (Sicinski et al., 1995). These, and other studies (Wang et al., 1994; Musgrove et al., 1993; Musgrove et al., 1994) suggest important functional links among progesterone, cyclin D1 and breast cancer and implicate the mitotic cell cycle in progesterone-dependent differentiation of the breast.

Opposing views that progesterone is a proliferative hormone in the breast are currently reflected in clinical practice (Cummings, 1991; Stanford et al., 1995; Colditz et al., 1995). Progestins are added to estrogens for hormone replacement therapy at menopause, because they block the proliferative and tumorigenic effects of unopposed estrogens in the uterus. However, women who have been hysterectomized are not given progestins to spare their breasts from the presumed proliferative effects of these hormones (Going et al., 1988; Potten et al., 1988; Anderson et al., 1989). This is defended by the prevailing notion that progesterone is differentiative in the uterus but proliferative in the breast (Clarke and Sutherland, 1990; Horwitz, 1992).

It is now clear that control of proliferation and differentiation are linked by events that occur in G1 of the cell cycle (Pardee, 1989; Steinman et al., 1994; Halevy et al., 1995; Parker et al., 1994). Recent studies implicate upregulation of cyclin-dependent kinase (cdk) inhibitors,

particularly p21 ($p21^{Kin1,Waf1,Sdi1}$) (Xiong et al., 1993), not only in inhibiting cell proliferation, but in promoting differentiation. In contrast, overexpression of cyclin D1 inhibits the differentiative program (Skapek et al., 1995) and in the breast, promotes cellular hyperplasia and tumor formation (Wang et al., 1994).

The molecular mechanisms underlying the proliferative and differentiative effects of progesterone at its target tissues have been difficult to assess for several reasons (Sicinski et al., 1995; Horwitz, 1992). First, in most progesterone target cells the levels of PR are regulated by estradiol (Horwitz and McGuire, 1978). Therefore, obligatory pretreatment with estradiol, itself a potent proliferative agent (Dickson and Lippman, 1995), confounds assessment of progesterone's role on growth and other cellular processes. Second, acutely, progesterone has dual effects on the cell cycle: it inhibits reentry of cells from mitosis into G1, but stimulates progression of cells through G1 (Musgrove et al., 1991). This complicates analysis of its role in proliferation. Third, progesterone target tissues contain two isoforms of PR, the 120 kDa B-receptors and the N-terminally truncated 94 kDa A-receptors (Horwitz and Alexander, 1983), that have unequal transcriptional activities (Tora et al., 1988; Meyer et al., 1992; Sartorius et al., 1994b). The two isoforms are dissimilarly regulated and expressed during development, following hormone treatments and in different target tissues and tumors (Spelsberg and Halberg, 1980; Boyd-Leinen et al., 1982; Kato et al., 1993; Graham et al., 1995). Our approach to reconciling these complexities, which limit studies of progesterone actions, has been to construct simpler model systems. To that end, we have used the $T47D_{CO}$ human breast cancer cell line, in which the two PR isoforms have escaped from estrogen controls and are constitutively expressed (Horwitz et al., 1982). These cells allow one to isolate the effects of progesterone and eliminate the confounding

effects of estradiol. To define the role of each receptor isoform, we recently isolated and subcloned a PR-negative subline of $T47D_{CO}$, and used it as the recipient into which either the B- or A-isoform of PR was stably reintroduced to produce T47D-YB and T47D-YA cells (Sartorius et al., 1994a).

We have used T47D-YB cells to analyze the role of progesterone on the factors that regulate cell-cycle progression and integrate growth regulatory signals. This includes measurement of the protein levels of key cyclins (Hunter and Pines, 1994; Sherr, 1994; King et al., 1994; Nurse, 1994; Heichman and Roberts, 1994); the levels and phosphorylation state of the retinoblastoma (Rb) tumor suppressor (Weinberg, 1995); and the levels of cdk inhibitors (Hunter and Pines, 1994; Peter and Herskowitz, 1994). Since T47D-YB cells are resistant to the proliferative effects of epidermal growth factor (EGF), we analyzed the role of cross-talk between the progesterone and EGF signaling pathways, on these proliferative events. We find that after stimulating one round of cell division, a single pulse of progesterone arrests T47D-YB cells in late G1 of the second cycle, by raising the levels of p21 and $p27^{Kip1}$. Additional progesterone augments the inhibition. However, during the arrested state, the cells acquire EGF sensitivity and resume cycling, during a window defined by falling p21 levels. We propose that progesterone establishes a restriction point that sensitizes its target cells to other signaling molecules, and argue that progesterone is neither inherently proliferative nor antiproliferative, but is a permissive hormone upon which secondary factors define the fate of the cell.

RESULTS

Progesterone treatment leads to growth resistance despite functional receptors. To study the proliferative effects of progestins, parallel sets of PR-negative Y cells (Figure 1, panel A), A-receptor containing YA cells (panels B,D) and B-receptor containing YB cells (panels C,E), were grown in control medium or in medium containing the following additions: the synthetic progestin agonist R5020 alone; the antiprogestins RU486 or ZK98299; or R5020 plus one of the antiprogestins. Sets of cells were harvested every 4 hrs for 48 hrs, and the percent in $S+G_2M$ for each treatment group was determined by flow cytometry. Optimum concentrations for each hormone were determined in preliminary studies. As shown in Figure 1, panel A, the percent of PR-negative Y cells in $S+G_2M$ during 50 hours of treatment with 30 nM R5020, 100 nM RU486 or 1 μg ZK98299 is no different than untreated controls, supporting the idea that proliferative responses to progestins are dependent on the presence of PR. These cells are also unresponsive to estradiol (not shown) and EGF (see below). Compared to controls, in either A-receptor-(panels B,D) or B-receptor-(panels C,E) containing cell lines, the percent of cells in $S+G_2M$ starts to climb approximately 12 hours after R5020 is added to the medium, peaks at 20-24 hours, and returns to basal levels approximately 36 hours later. During this transient increase in mitotic activity, the percent of cells in $S+G_2M$ rises from basal levels of 15-20% in the untreated controls, to 45-50% in the R5020-treated sets. The two antiprogestins, RU486 or ZK98299, when each is given alone, have minor effects in the two cell lines. There is a consistent trend for the antagonists to have a transient growth suppressive effect in the YA but not the YB cells. However, in either cell line, the two antiprogestins completely block the R5020-induced proliferative burst, suggesting again that the proliferative effects of progestins are PR-dependent. Since the two cell lines did not differ significantly, the remaining studies described here used

T47D-YB cells. Control of proliferation by antiprogestins in the presence of cAMP does exhibit PR isoform specificity (in preparation).

Although R5020 is known to be poorly metabolized in $T47D_{CO}$ cells (Horwitz et al., 1986), it was theoretically possible that the transitory nature of the proliferative increase seen in Figure 1 was due to degradation of the hormone during the 40 or more hours of cell culture. To determine if additional hormone would produce a sustained rise in proliferation, T47D-YB cells were treated with R5020 at time 0, then given a second pulse of R5020 at 48 hours, and the percent of cells in different phases of the cell cycle was measured for 96 hours (Figure 2A). Surprisingly, while the initial proliferative rise at 20 hours was clearly evident, the cells were completely refractory to the second hormone challenge (gray arrow).

We postulated that the resistance to additional R5020 at 40 hours was due to receptor down-regulation produced by the continuous exposure to hormone. Indeed, while untreated control cells have high levels of B-receptors as measured by immunoblotting (Figure 2B), 10 hours after the start of R5020 treatment, B-receptors are barely detectable. If the cell culture medium is not changed, there is little recovery for at least 50 hours. However, as shown in Figure 2B, following progesterone treatment, B-receptor down-regulation is transitory -- receptor levels are depressed 10 hours after the hormone addition, but they rapidly replenish to control levels, because this natural ligand is metabolized in $T47D_{CO}$ cells with a half-life of 2-4 hours (Horwitz et al., 1986). We therefore postulated that T47D-YB cells are refractory to a second hormone challenge after initial treatment with R5020 (Figure 2A) because PR are still down-regulated at 40 hours, and we reasoned, that if the cells were initially treated with progesterone their progestin-sensitivity would be restored at 40 hours. Figure 2A shows that this is not the case. Like R5020,

progesterone produces a transient increase in cell proliferation. Nevertheless, 40 hours after the initial progesterone-induced growth, and despite adequate levels of receptors (Figure 2B), the cells are completely refractory to subsequent treatment with R5020.

This result was surprising, and we wondered whether the replenished receptors were somehow functionally incompetent (Figure 3). To test this, we measured the ability of replenished receptors to activate transcription of chloramphenicol acetyl transferase (CAT) driven by a PR-responsive promoter. Parallel sets of YB cells were pretreated with progesterone at zero time. Twenty-four hours later a subset of cells was transfected with the PRE_2-$TATA_{tk}$-CAT reporter, in which the proximal promoter of the thymidine kinase gene is controlled by two upstream progesterone response elements (PRE). The cells were glycerol shocked at 46 hours to complete the transfection and immediately treated with a second dose of either progesterone or R5020 for an additional 24 (Figure 3, inset) or 48 hours (not shown) before they were harvested, and CAT activity was measured in cell lysates. A control set of transfected cells was left untreated at 40 hours (Figure 3, inset). Additionally, parallel sets of untransfected cultures were treated with a second pulse of R5020 at 40 hours and the cells were harvested periodically over the next 48 hours for analysis of cell-cycle phases by flow cytometry (Figure 3). As shown above, following the initial progesterone-induced proliferative burst, the cells enter a period of growth arrest lasting at least 80 hours, from which they cannot be rescued by retreatment with R5020 at 40 hours (open boxes). However as the inset (Figure 3) shows, this inhibition is not due to incompetent receptors, since they can induce CAT transcription in the same endocrine setting. Thus, despite progesterone pretreatment at time zero, at 40 hours R5020 and progesterone strongly induce transcription from the PRE-$TATA_{tk}$-CAT reporter. The control cells that

received no hormone at 40 hours confirm, that 40 hours after the initial progesterone dose, insufficient hormone remains in either the cells or the medium to transactivate the promoter, so that the high levels of CAT activity seen in the 40 hour R5020 and progesterone treated sets must be due to the second hormone challenge. We conclude that the replenished receptors are fully functional: they are capable of binding ligand, of binding DNA at cognate PREs, and of interacting with the requisite factors on a promoter to activate transcription (Beato et al., 1995).

Progesterone produces G1 phase arrest by up-regulating p21 and $p27^{Kip1}$. Since recent studies suggest links among progesterone, breast cancer and the cell cycle (Lydon et al., 1995; Sicinski et al., 1995; Wang et al., 1994; Musgrove et al., 1993; Musgrove et al., 1994), we measured the protein levels of cell cycle regulatory proteins during the dual progestin treatment regimen, in an attempt to explain the progesterone resistance. Figure 4 shows the changes in protein levels over a 70 hour period, of cyclins D1, D3 and E, of the inhibitors p21 and $p27^{Kip1}$, and of Rb. These proteins regulate progression of cells through G1 (Hunter and Pines, 1994; Sherr, 1994). The cells were treated with progesterone at time zero (arrow), followed 40 hours later (arrow) either by no treatment (closed boxes) or by R5020 (open boxes). The cells were harvested periodically and aliquots of cell lysates were assayed for the cell cycle proteins by immunoblotting, and for the percent of cells in $S+G_2M$ by flow cytometry. In Figure 4, the flow cytometric data are shown by the dashed line, since they represent the same data points that are shown in detail in Figure 3. Following treatment with progesterone, the levels of cyclin D1 rise transiently and then fall, coincident with the increased proliferative activity observed in the first 24 hours. D1 expression increases within 2 hours of progesterone addition (not shown). A second abortive peak is observed between 25-40 hours, and levels then fall again after 40 hours. This

second peak is not always observed; it is markedly attenuated if the basal cell proliferation rate is relatively low. Note that the study shown in Figure 4 involves cells that have a rapid basal proliferation rate, characteristically seen in late passages. The changes in D3 levels resemble those of D1, in which an initial rise is followed by a persistent fall. If cells are retreated with R5020 at 40 hours there is an abortive rise in D3 levels (open boxes). Cyclin E levels fall to low levels as the cells transit the cell cycle in the first 24 hours after progesterone, then rise spontaneously to very high levels and remain high, in cells that receive no further treatment. This lack of cyclin E degradation suggests that the cells are blocked in late G1 and cannot enter S-phase (Sherr and Roberts, 1995). Retreatment of the cells with R5020 at 40 hours only minimally lowers cyclin E levels, and the flow cytometry data confirm that these cells do not resume cycling.

Because progesterone produces a prolonged growth refractory state, protein levels of cyclin kinase inhibitors were also measured (Figure 4). We find that $T47D_{co}$ cells and their descendents do not express p16 ($p16^{INK4,MTS1}$) (Serrano et al., 1993), but they express p21 ($p21^{Cip1,Waf1,Sdi1}$) (Xiong et al., 1993) and $p27^{Kip1}$ ($p28^{lck1}$) (Polyak et al., 1994). A rise in protein levels of the inhibitors begins as cyclin levels are declining. The levels of p21 increase first, peak approximately 36 hours after the initial progesterone pulse, and fall. This decline can be delayed ~18 hours by retreatment of the cells with R5020 at 40 hours. Thus elevated p21 levels are transiently stabilized by the second progesterone pulse. The levels of $p27^{Kip1}$ remain relatively unchanged for 36 to 40 hours after the initial progesterone treatment, and then start to climb as the cells arrest. Its levels eventually fall after 70-80 hours (not shown here, but see Figure 7D) in the absence of a second hormone pulse, as the cells spontaneously recover their ability to

proliferate. However, additional hormone produces even higher and sustained levels of $p27^{Kip1}$ resulting in persistent growth suppression (see Figure 6B).

Since the signals from the G1 cyclins and the cdk inhibitors are integrated into the Rb transcriptional regulatory pathway (Weinberg, 1995), the protein levels of Rb and its phosphorylation state were measured at 6 hour intervals for 72 hours following the two hormone pulses (Figure 4, immunoblot). In the first 12 to 18 hours, levels of Rb are high but the protein is predominantly in its inactive hyperphosphorylated state. In this period, lacking sufficient amounts of inhibitory Rb, the cells undergo one round of cell division. However, coincident with the G1 arrest in the second cycle, 24 hours after progesterone treatment, significant levels of the hypophosphorylated (arrow) active repressor form of Rb are present. Additionally, total Rb protein levels down-regulate by more than 90%, consistent with cell cycle arrest in G1 (Xu et al., 1991). This decline occurs in the presence (not shown) or absence (Figure 4) of a second hormone pulse at 40 hours.

The profound quality of the arrest in G1 is confirmed by analysis of cyclin A and B levels as shown in Figure 5. These cyclins are produced in S- and G2-phase respectively, in preparation for mitosis (King et al., 1994; Nurse, 1994; Heichman and Roberts, 1994). In progesterone treated T47D-YB cells, the levels of both cyclins reach a peak during the initial proliferative burst, but then fall precipitously to almost undetectable levels for at least 80 hours (not shown) and they cannot be rescued by a second hormone pulse at 40 hours (arrow) as shown in Figure 5.

When do T47D-YB cells regain sensitivity to progestins? To assess this (Figure 6A), parallel sets of cells were treated with progesterone at time zero, then exposed to R5020 starting

48 hours later, or every six hours thereafter. For each set of time-points, the ability of R5020 to induce proliferation was monitored for the subsequent 36 hours. Each hormone-treated set was compared to control cells that had not received the second R5020 dose to monitor spontaneous recovery. Figure 6 shows that resistance persists until approximately 72 hours (open triangles) after the initial progesterone dose, at which point R5020 produces a brisk proliferative response (Figure 7A), coincident with spontaneous recovery (not shown, but see Figure 7C). Thus, only when they are poised to resume growing spontaneously (see Figure 7C), do the cells regain sensitivity to progestins. The extent and duration of this second proliferative burst resembles that of the initial response seen in naive, hormone-untreated cells (Figures 1 and 2). This recovery time varies somewhat among experiments, depending on the basal cell proliferation rate at time zero; cells with a high basal rate, usually late passage cells, recover more quickly. In general T47D-YB breast cancer cells remain in stasis for ~3 days after a brief pulse of progesterone (recall the t½ is 2-4 hours), but then resume growing at the same rate as controls (Figure 6B). On the other hand, continuous exposure to progesterone or R5020 every 48 hours produces permanent growth arrest (Figure 6B). This arrest is associated with a 12 to 15-fold increase in the levels of p21 and a 6 to 7-fold increase in the levels of $p27^{Kip1}$ (Figure 6C).

EGF Induces a Proliferative Response in Progesterone Resistant Cells. To determine if, during their progesterone-resistant state, T47D-YB cells are resistant to other mitogenic signals, we tested the effects of EGF. This mitogen is an important growth factor in breast cancers, and clinically, an inverse relationship exists between steroid receptor loss (with concomitant hormone resistance) and the expression of EGF receptors (Dotzlaw et al., 1990; Chrysogelos and Dickson, 1994; Fox et al., 1994). We therefore tested the relationship, if any,

between progesterone resistance and EGF growth sensitivity (Figure 7A-D). Control T47D-YB cells express functional EGF-receptors, and a 10 nM pulse of EGF strongly induces mitogen activated protein (MAP) kinase activity (not shown). However, the cells are resistant to the growth stimulatory effects of EGF as shown in Figure 7A. In this study, cells that had received no prior treatment were incubated with 10 nM recombinant human EGF or left untreated, and the percent of cells in $S+G_2M$ was measured every 6 hours for 30 hours (Figure 7A). As shown, the proliferation of cells treated acutely with EGF did not differ significantly from controls.

To determine whether chronic EGF treatment affected growth, cells received EGF continuously for 6 days in the presence or absence of continuous R5020, and their proliferation rate was compared to that of untreated or R5020-treated cells (Figure 7B). EGF alone does not accelerate growth above the control rate and it cannot relieve the growth suppression produced by continuous R5020.

Surprisingly therefore, T47D-YB cells can be sensitized to the proliferative effects of EGF by a brief progesterone pulse (Figure 7C). In this study, sets of YB cells that had been pretreated with progesterone at time 0 were challenged with EGF at various time-points, starting at 36 hrs. Control sets received no second treatment, to monitor spontaneous recovery from the progesterone-induced arrest. While EGF given 36 hrs after progesterone was ineffective, the cells acquire sensitivity to EGF starting approximately 46 hrs after progesterone pretreatment, and exhibit an extensive proliferative burst following a 6 hour lag. Cells treated with EGF 52 hours after progesterone respond even faster (Figure 7C). Recall that at these time-points the cells are insensitive to progesterone (Figure 6) and have not recovered spontaneously (Figure 7C, dashed line). These, and other studies (not shown) suggest that there is a critical period during

progesterone-induced growth arrest, in which T47D-YB cells acquire sensitivity to the proliferative effects of EGF, which accelerates their reentry into the cell cycle.

What accounts for this brief sensitivity to EGF? Figure 7D shows that following the progesterone pulse, p21 levels rise, peak at ~30 hours and then fall by 36 hours; at a time when $p27^{Kip1}$ levels are rising. These data suggest that the relative levels of the cdk inhibitors, particularly falling levels of p21, influence cell sensitivity to EGF. This pattern occurs only following the brief signal produced by the short half-life of one progesterone pulse. More prolonged progestin exposure, produced either by retreatment with progesterone or by treatment with a poorly metabolizable synthetic progestin like R5020, prevents or slows the fall in p21 levels (Figure 4) so that this event overlaps more extensively with the rise in $p27^{Kip1}$. As a result EGF cannot overcome a continuous progestin signal as shown in Figure 7B.

DISCUSSION

We demonstrate here, under conditions in which the proliferative actions of progesterone can be isolated from those of other mitogens, and in which PR levels are autonomously controlled, that this steroid hormone elicits a single round of mitosis in breast cancer cells, following which the cells chronically arrest in late G1 of the second cycle in a persistent progesterone resistant state. Clearly, the cellular conditions that unmasked this property of progesterone are artificial (Sartorius et al., 1994a). Nevertheless, they begin to allow study of a hormone whose proliferative effects have been intensely disputed in part because of their complexity (Clarke and Sutherland, 1990). We believe that by simplifying the model we can dissect out the unique role that progesterone plays in physiological systems in which its actions are otherwise intricately regulated by cross-talk with other steroid hormones and growth factors.

Progesterone is autoinhibitory. The immediate response of proliferating breast cancer cells to progesterone is an acceleration of the cell cycle driven by increased levels of cyclins D1, D3 and E, and by accumulation of inactive forms of Rb in the face of persistent low steady-state levels of p21 and $p27^{Kip1}$. Under these conditions cyclin D-cdk4 complexes accumulate above the inhibitory threshold of $p27^{Kip1}$ (Kato et al., 1994), allowing the cells to progress past the G1 restriction point and enter mitosis. Because the breast tumor cells used in these studies are proliferating rapidly initially (at a "low" proliferation rate, 15-25% of cells are in S+G2M at any time) and progesterone more than doubles this rate (leading to more than 50% in S+G2M), the cells proceed through mitosis and re-enter G1 of the second cycle in a partially synchronized state. At this point however, the cells are unable to adequately replenish the depleted stores of cyclin Ds, Rb protein levels are extensively down-regulated, cyclin E levels rise sharply, followed first by

rising p21, and later by rising $p27^{Kip1}$ levels. Cyclin A, required for progression through S-phase and cyclin B, the primary mitotic kinase, are completely down-regulated. Taken together these data indicate that the cells are arrested late in G1 (Sherr and Roberts, 1995).

One surprising finding is that additional progesterone cannot override the growth suppression produced by the first progesterone dose, despite the presence of adequate levels of transcriptionally competent PR. Thus, the proliferative block is not at the level of the PR signaling system. In fact, the growth arrest may require the presence of functional PR since it appears to be due to sustained upregulation of p21 and $p27^{Kip1}$, produced by a positive feed-back loop initiated by at least two progestin treatments given prior to the time at which the cells would spontaneously recover. Thus, we believe that sustained progesterone is autoinhibitory, in contrast to transient progesterone which is stimulatory. This model has important implications for the scheduling of progestin treatments in clinical settings, since it predicts that the effects of continuously administered progestins (Sedlacek and Horwitz, 1984) differ significantly from those of episodically or cyclically administered progestins (Going et al., 1988; Anderson et al., 1989); the former would be growth inhibitory and the latter stimulatory. These data also suggest that the cyclical progesterone of the menstrual "cycle" can have different physiological consequences than the continuous progesterone of pregnancy. A model in which the rate and duration of progesterone treatment controls the type of response would reconcile contradictory views that this hormone is either proliferative or differentiative. A similar model, in which proliferation or differentiation is controlled by the duration of MAPK signaling pathway was recently described (Marshall, 1995).

Progesterone enhances EGF sensitivity. Failure of progestins to reinitiate proliferation after a single progesterone pulse is also not due to insensitivity of the cell cycling machinery, since an alternate mitogenic signal emanating from the cell surface can transiently reactivate proliferation. The mechanisms by which progesterone sensitizes the cells to the proliferative effects of EGF remain unclear. Untreated T47D-YB cells do not respond to the proliferative signals of EGF (Figure 7A), although they express immunoreactive EGF receptors, respond to EGF by activating p42 and p44 MAP kinases and are descendent from PR-positive $T47D_{co}$ cells in which EGF stimulates growth without progesterone pretreatment (not shown).

A model that may explain EGF-dependent proliferation after a single pulse of progesterone, but resistance after a second progesterone pulse or during continuous progesterone treatment, is outlined in Figure 8. We have shown that one progesterone pulse leads to a transient rise in p21 levels and that $p27^{Kip1}$ levels start to rise as p21 levels are falling. Therefore, after a single progesterone pulse, there is a transient window of EGF responsiveness (at a time that varies depending on basal cell proliferation rates) defined at the start by relatively low p21 levels and at the end by the spontaneous resumption of cell growth (Figure 7C). However, a second dose of progesterone delays the fall in p21 and increases the levels of $p27^{Kip1}$ (Figure 4), eliminating EGF responsiveness. Thus proliferative sensitivity to EGF is dependent on the cell cycle state and progestational history of the cells. This model predicts that the transient proliferative effect of EGF would be blocked by co-administration of progesterone during the EGF challenge, and conversely that, with appropriate timing, antiprogestins could enhance cellular sensitivity to EGF. Experiments to test these hypotheses are in progress.

There is considerable evidence linking the EGF and progesterone signaling pathways in breast cancer. This includes attenuation of progestin responsiveness and decreases in PR levels in cells treated with EGF (Sarup et al., 1988); augmentation of the proliferative, differentiative and transcriptional effects of progestins by cotreatment with EGF (Modiano et al., 1991; Haslam et al., 1993; Krusekopf et al., 1991); and progestin-specific regulation of EGF and EGF receptor levels (Sarup et al., 1988; Dotzlaw et al., 1990; Murphy et al., 1991). There are also provocative clinical data linking enhanced expression of EGF receptors to acquisition of steroid hormone resistance in breast cancer (Chrysogelos and Dickson, 1994; Fox et al., 1994).

Is progesterone proliferative or differentiative? Proliferation and differentiation are complex processes governed by the concerted activity of multiple regulatory factors. Both processes appear to have the common requirement that cells stop in G1 to await appropriate directional signals (Steinman et al., 1994; Halevy et al., 1995; Parker et al., 1994). Our demonstration that progesterone can advance cells to this check-point may reconcile opposing views that progesterone is either a proliferative or a differentiative hormone. We suggest that progesterone is neither, but that it is a competency factor necessary to drive cells into either pathway, and that breast cell proliferation and differentiation are intricately connected. In this model, progesterone accelerates cells to the G1 check-point in the second cycle, whereupon other, possibly tissue-specific factors, determine the fate of the cell. Therefore the final state of progesterone target tissues is determined by cross-talk between progesterone and growth or differentiative factors that remain to be defined.

Experimental Procedures

Cell Lines and Reagents.

Wild-type PR-positive $T47D_{co}$ breast cancer cell lines, their monoclonal PR-negative T47D-Y derivatives, and T47D-Y cells stably expressing either A- or B-receptors (T47D-YA or T47D-YB cells) were previously described (Sartorius et al., 1994a). Cells are routinely cultured in 75 cm^2 plastic flasks or 6-well multiwell plates and incubated in 5% CO_2 at 37°C in a humidified environment. The stock medium consists of Eagle's Minimum Essential Medium with Earle's salts (MEM), containing L-glutamine (2mM) buffered with sodium bicarbonate (4 μg/L) and HEPES (4.8 μg/L), insulin (6 ng/ml) and 5% fetal calf serum (FCS, Hy-Clone) without antibiotics. For routine subculturing, cells are diluted 1:20 into new flasks once per week, and medium is replaced every 2-3 days. Cells are harvested by incubation in Hank's EDTA for 15 min at 37°C.

Antibodies were obtained from the following sources: anti-cyclin A, -cyclin B1, and -cyclin D1 were from Upstate Biotechnology, Lake Placid, NY; anti-cyclin D3 and E were from Pharmingen, San Diego, CA; anti-p21 and anti-$p21^{Kip1}$ were from Santa Cruz Biotechnology, Santa Cruz, CA; anti-pRb was a gift from Wen-Hwa Lee (University of Texas Health Science Center, San Antonio); anti-PR AB52 and B30 were produced in our laboratories (Estes et al., 1987); and horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad Laboratories, Hercules, CA.

Flow Cytometry

$2x10^5$ cells were plated into duplicate wells of 6-well plastic dishes with 3 ml of serum-containing medium. After 24 hours, progesterone, or the synthetic agonist R5020 (Roussel-Uclaf, France) or the antiprogestins RU486 (Roussel-Uclaf, France) or ZK98299 (Schering AG, Germany) were added in ethanol, at final concentrations of 30 nM, 100 nM or 1 μM, respectively. Control medium contained only ethanol. Some cells received 60 ng/ml (10 nM) human recombinant EGF (Upstate Biotechnology, Inc., Lake Placid, NY).

Cells were harvested at the start of treatment (control, zero time) and every 4 or 6 hours after hormone addition, into 1 ml of Hank's EDTA and vigorously pipetted. The cell suspension was pelleted, resuspended into 1 ml of Krishan's stain (Krishan, 1975) containing propidium iodide and RNase and again vigorously pipetted. Samples were cooled to 4°C, and 10,000 cells were analyzed on an Epics 752 flow cytometer (Coulter Electronics, Hialleah, FL), using an incident beam from an argon laser at 488 nm, 500 mW. The cells were gated on forward angle vs. 90° light scatter to eliminate cellular debris and doublets. Red fluorescence, corresponding to DNA, was collected through a 590 nm longpass filter and histograms of DNA content vs. cell number were constructed. Cell cycle analyses of the DNA histograms were performed using the ModFit Analysis program (Veritey Software House, ME) which provides fits for the G_O/G_1, S and G_2 M fractions of the population. The S- and G_2 M-phase fractions were combined into a single "growth fraction". In some figures, the percent of cells in S+G_2 M in the hormone-treated sets were compared to the percent of cells in S+G_2 M in the untreated controls. For long-term growth studies, cells were harvested into 1 ml of Hank's EDTA, pipetted vigorously to obtain single cell suspensions, and aliquots were counted using a hemocytometer.

Immunoblotting

For measurements of PR, whole cell extracts were prepared in 0.4M KCl as previously described (Sartorius et al., 1994a; Sartorius et al., 1994b). Receptors were resolved by electrophoresis on an 11% polyacrylamide gel containing sodium dodecyl sulfate (SDS), and then transferred to nitrocellulose. After incubation with anti-PR monoclonal antibodies AB-52 and B-30 (Estes et al., 1987), the receptor bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL). For cell cycle proteins, cells were harvested at 50-80% confluence and washed in phosphate-buffered saline. Aliquots were removed for analysis by flow cytometry to simultaneously determine cell cycle distribution. The remaining cells were resuspended in Laemmli sample buffer (Laemmli, 1970) at 1-4x10^7 cells per ml, immediately boiled for 5 minutes, sheared through a syringe needle to reduce viscosity, aliquoted and stored at -80°C. Volumes of cell extracts normalized to ~50 μg of total protein as measured by Ponceau S were subjected to gel electrophoresis. Proteins were transferred for 45 minutes at 0.5 A to Immobilon P membranes (Millipore) using a Genie Electroblotter (Idea Scientific, Minneapolis). After incubation with the appropriate antibodies, protein bands were detected by enhanced chemiluminescence (Amersham). Film exposures ranged from 2 seconds to 1 hr depending on the primary antibody. Bands were quantitated using a digital scanner and the Image program (National Institutes of Health), and normalized to levels of pSTAIRE sequence-containing cdks (Branbilla and Draetta, 1994).

Transfection and Transcription

T47D-YB cells were plated and grown in 100 mm^2 cell culture plates in MEM supplemented with 5% FCS. Progesterone (30 nM) was added 40 hrs prior to completion of transfec-

tion. Transfection of plasmid DNA into cells was performed 24 hours after the start of progesterone treatment by calcium phosphate coprecipitation using 1 μg of the progesterone response element $(PRE)_2$-$TATA_{tk}$-chloramphenicol acetyl transferase (CAT) reporter (Sartorius et al., 1994b), 3 μg of the β-galactosidase expression plasmid PCH110 (Pharmacia) to correct for transfection efficiency, and 15 or 16 μg of Bluescribe carrier plasmid (Stratagene) for a total of 20 μg DNA, as previously described (Sartorius et al., 1994b). Sixteen hours later transfection was completed when the medium was aspirated, and the cells were shocked at room temperature for 4 minutes with 5 ml of Hank's Balanced Salt Solution containing 20% glycerol. After washing the cells twice with 10 ml of serum-free MEM to remove the glycerol, 10 ml of MEM containing 5% FCS were added per dish, either without or with 30 nM progesterone or R5020. Cells were harvested after an additional 24 or 48 hours. Cells in duplicate plates were lysed by freeze-thawing in 200 μl of 0.25M Tris, pH 7.8. Lysates (50 μl) were assayed for β-galactosidase activity, and normalized aliquots were assayed for CAT activity by thin layer chromatography (TLC) as described (Sartorius et al., 1994b).

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Figure Legends

Figure 1. The synthetic progestin R5020 induces a proliferative burst in PR-positive cells that is inhibited by antiprogestins.

(A) PR-negative T47D-Y cells were treated with the agonist R5020 (■) or the antiprogestins RU486 (●) or ZK98299 (O) for 48 hours. Cells were harvested every 6 hours and the percent of cells in S+G_2 M was measured by flow cytometry. (B) T47D-Y cells stably expressing A-receptors (YA) were treated with R5020 (■), RU486 (●) or both (O), and cells in S+G_2 M were measured by flow cytometry over a 48 hour period. (C) T47D cells stably expressing B-receptors (YB) were treated with R5020 (■), RU486 (●) or both (O). (D) Same as B except that ZK98299 was the antiprogestin. (E) Same as C except that ZK98299 was the antiprogestin. Change in % of cells in S+G_2 M is compared to controls, set at zero. In this study ~15-20% of control cells were in S+G_2 M.

Figure 2. After the initial proliferative burst, T47D-YB cells are resistant to another progestin challenge despite adequate PR levels.

(A) T47D-YB cells were untreated or were treated with progesterone (■) or R5020 (●) at 0 time, and again with R5020, 48 hours later. Cells were harvested every 4-6 hours for 96 hours. Percent cells in S+G_2 M were measured by flow cytometry. Changes in the percent S+G2/M of hormone-treated cells are compared to untreated controls, which were set at 0. (B) T47D-YB cells were treated with R5020 or progesterone at 0 time and harvested periodically for 50 hours. B-receptor levels were measured by immunoblotting with anti-PR antibodies; hormone-untreated controls are shown at time 0.

Figure 3. T47D-YB cells resistant to the proliferative effects of progestins, contain transcriptionally competent hormone responsive PR.

T47D-YB cells were treated with progesterone at time 0, and retreated with progesterone at 40 hours (□), or left untreated (■). Cells were harvested every 4-6 hours for 80 hours and the percent of cells in $S+G_2$ M were measured by flow cytometry.

Inset. YB cells treated with progesterone at time 0 were transfected with PRE_2-$TATA_{tk}$-CAT reporter at 46 hours and left untreated (–), or treated with R5020 or progesterone. Cells were harvested 24 hours later, lysates were prepared, normalized to β-galactosidase activity, and CAT activity was measured by thin layer chromatography. Duplicates from separate transfections are shown.

Figure 4. Progesterone treatment arrests T47D-YB cells in G1 of the second cycle by up-regulating p21 and $p27^{Kip1}$.

Growth, cyclin and cdk inhibitor levels. T47D-YB cells were treated with progesterone at time 0 (black arrow), and again at 40 hours (□) or left untreated (■) as shown in Figure 3. Cells were harvested every 4-6 hours for 80 hours and the percent in $S+G_2$ M was measured by flow cytometry (dashed lines; see Figure 3). Parallel sets of cells were lysed in Laemmli buffer, extracts normalized to total protein were resolved by SDS-gel electrophoresis and immunoblotted with antibodies to the cyclins and cdk inhibitors shown. Protein bands were detected by enhanced chemiluminescence and quantitated by densitometry and their levels were normalized to pSTAIRE levels determined in parallel.

Rb Immunoblot. T47D-YB cells were treated with progesterone at time 0 and parallel sets were harvested every 6 hours for 72 hours. Cells were lysed in Laemmli buffer, extracts were normalized to total protein level, resolved by SDS-PAGE and immunoblotted with an antibody directed against Rb.

Figure 5. Down-regulation of cyclin A and cyclin B after progesterone-induced growth-arrest.

T47D-YB cells treated with progesterone at 0 time (■) and again at 40 hours (□) as described in Figure 3, were harvested at the times indicated, lysed in Laemmli buffer, resolved by SDS-gel electrophoresis and immunoblotted with antibodies directed against cyclin A or cyclin B (■). Percent of cells in S+G2/M are indicated by the dashed line, from the same sets shown in detail in Figure 3. The highest cyclin protein level at any time-point was set at 100%.

Figure 6. A brief progesterone pulse leads to prolonged progesterone resistance accompanied by elevated levels of cdk inhibitors.

A. Time-course for restoration of progestin responsiveness after a brief progesterone pulse. Parallel sets of T47D-YB cells were treated with progesterone at time 0 (not shown), and 48 hours later, or every 6 hours thereafter up to 78 hours later, they were again treated with progesterone. The ability of the second progesterone dose to induce a proliferative response was monitored for the subsequent 36 hours. Cells were harvested at the time-points shown and percent in S+G2/M were measured by flow cytometry and were compared to control cells that had received no second hormone treatment (not shown).

B. Effects of single *vs.* daily progestin treatments on cell proliferation. Parallel sets of T47D-YB cells were untreated (■), treated once with progesterone at time 0 (●), or treated with progesterone (○) or R5020 (□) daily. Cells were harvested as shown over a 6 day period and the average number of cells, in duplicate flasks, were counted.

C. Levels of p21 and $p27^{Kip1}$ after chronic progesterone. Sets of cells from the chronic progesterone treatment (B, above) were harvested and lysed in Laemmli buffer. Lysate concentrations were normalized to total protein levels, resolved on SDS-PAGE, immunoblotted with anti p21 or anti $p27^{Kip1}$ antibodies, and relative cdk inhibitor levels were quantitated by densitometry and normalized to pSTAIRE levels determined in parallel blots.

Figure 7. Progesterone arrested cells can mount a transient proliferative response to EGF.

A. EGF effects on naive T47D-YB cells. T47D-YB cells growing under control conditions without progesterone, were treated with 10 mM recombinant human EGF at time 0. Cells were harvested every 6 hours and the percent of cells in S+G2/M was measured flow cytometrically and compared to EGF untreated controls, set at 0.

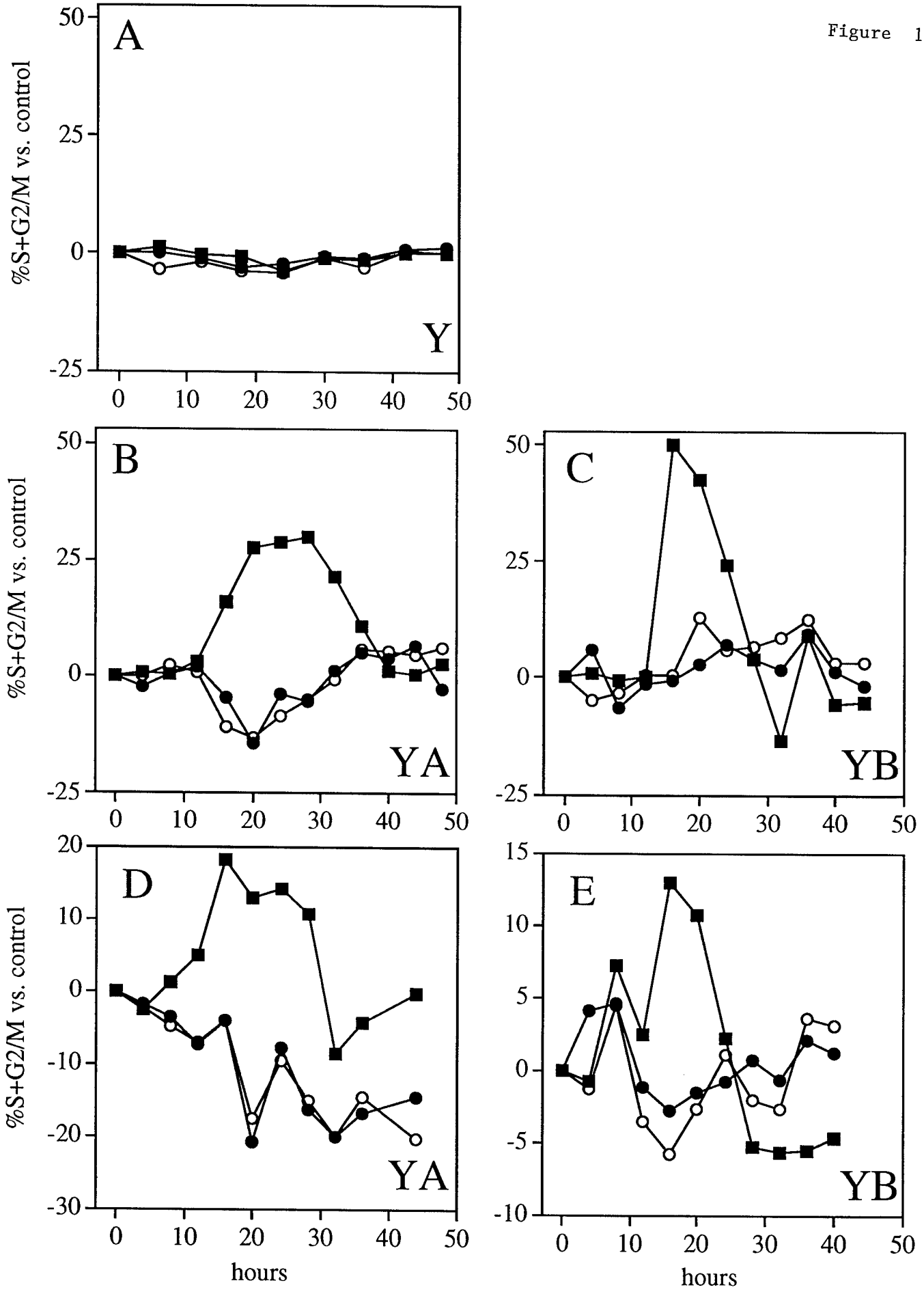
B. Chronic EGF and progestins. Parallel sets of T47D-YB cells were treated daily for 6 days with R5020 only (□), R5020 + EGF (○), EGF only (●) or left untreated (■). Cells were harvested daily and their number counted.

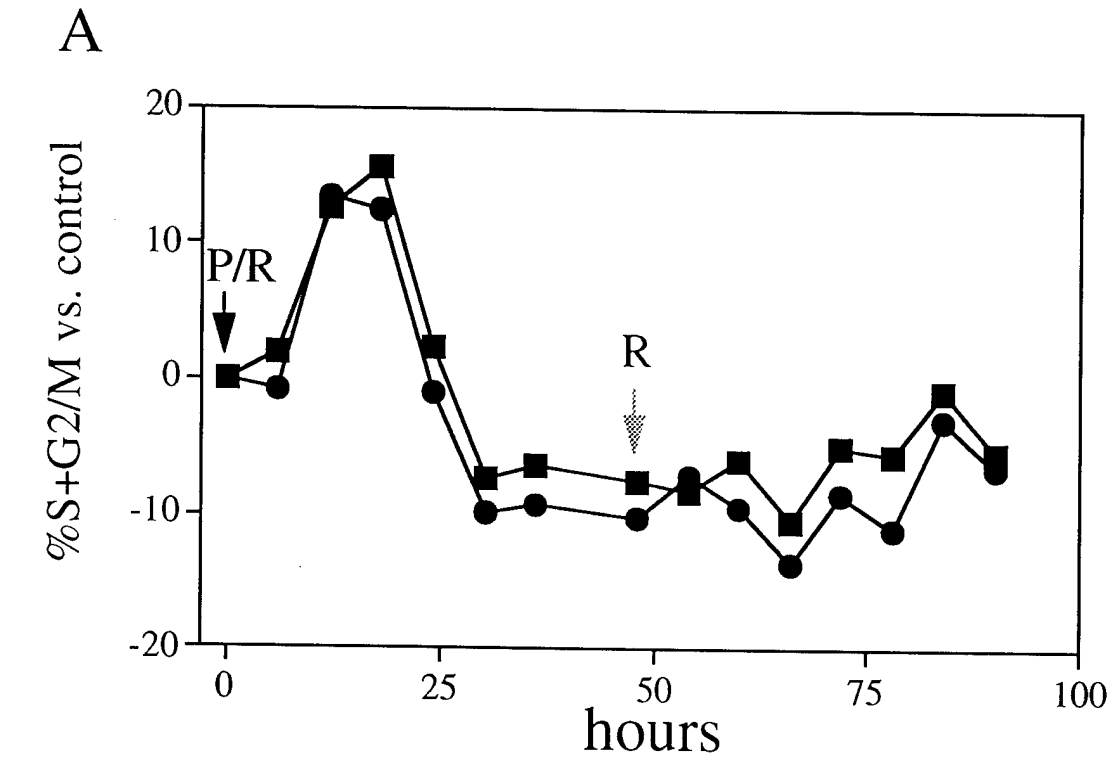
C. Proliferative effects of EGF after a pulse of progesterone. Parallel sets of T47D-YB cells were treated with progesterone at time 0. Control cells were left untreated for the subsequent 80 hours (dashed lines). Parallel sets received 10 nM EGF starting at 36, 46, 52, 58 and 64 hours after time 0, and the percent of cells in S+G2/M was monitored flow cytometrically over the subsequent 36 hours for each treatment group.

D. p21 and $p27^{Kip1}$ levels after a pulse of progesterone. Cells from set C, that received progesterone at time 0 and no further treatment, were harvested periodically as shown and lysed in Laemmli buffer. The lysates were normalized to total protein levels, resolved by SDS-PAGE, immunoblotted with anti p21 and anti $p27^{Kip1}$ antibodies, and levels of the inhibitors were determined densitometrically and normalized to pSTAIRE levels in the same lysates. The data were plotted as a percent of the maximum level for each inhibitor.

Figure 8. A model describing the effects of one (solid line) or two (dashed line) pulses of progesterone on levels of the cdk inhibitors p21 (thick line) and $p27^{Kip1}$ (thin line). The shadowed area represents a period of responsiveness to the proliferative effects of EGF produced by the single progesterone dose (P1) but blocked by the second dose (P2).

Figure 1


A
Y
B
YA
C
YB
D
YA
E
YB
%S+G2/M vs. control
%S+G2/M vs. control
%S+G2/M vs. control
hours
hours


A
20
10
0
-10
-20
%S+G2/M vs. control
P/R
R
0
25
50
75
100
hours

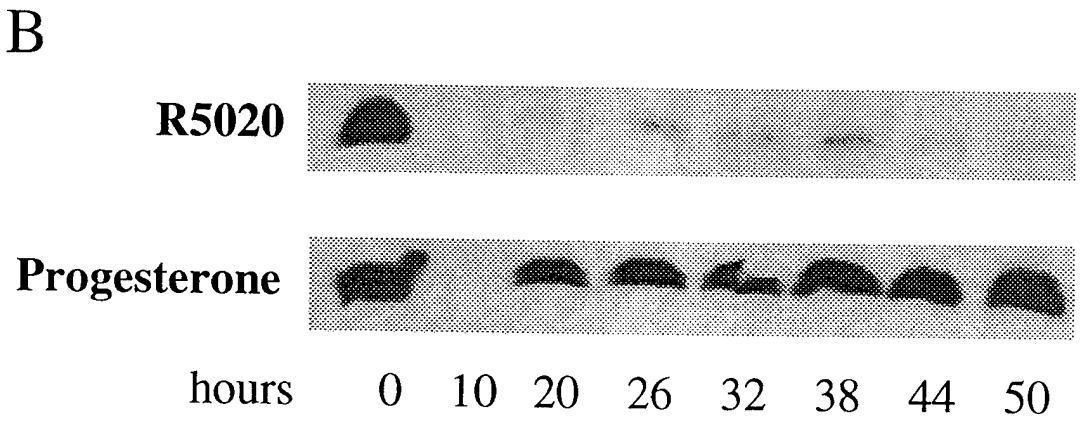
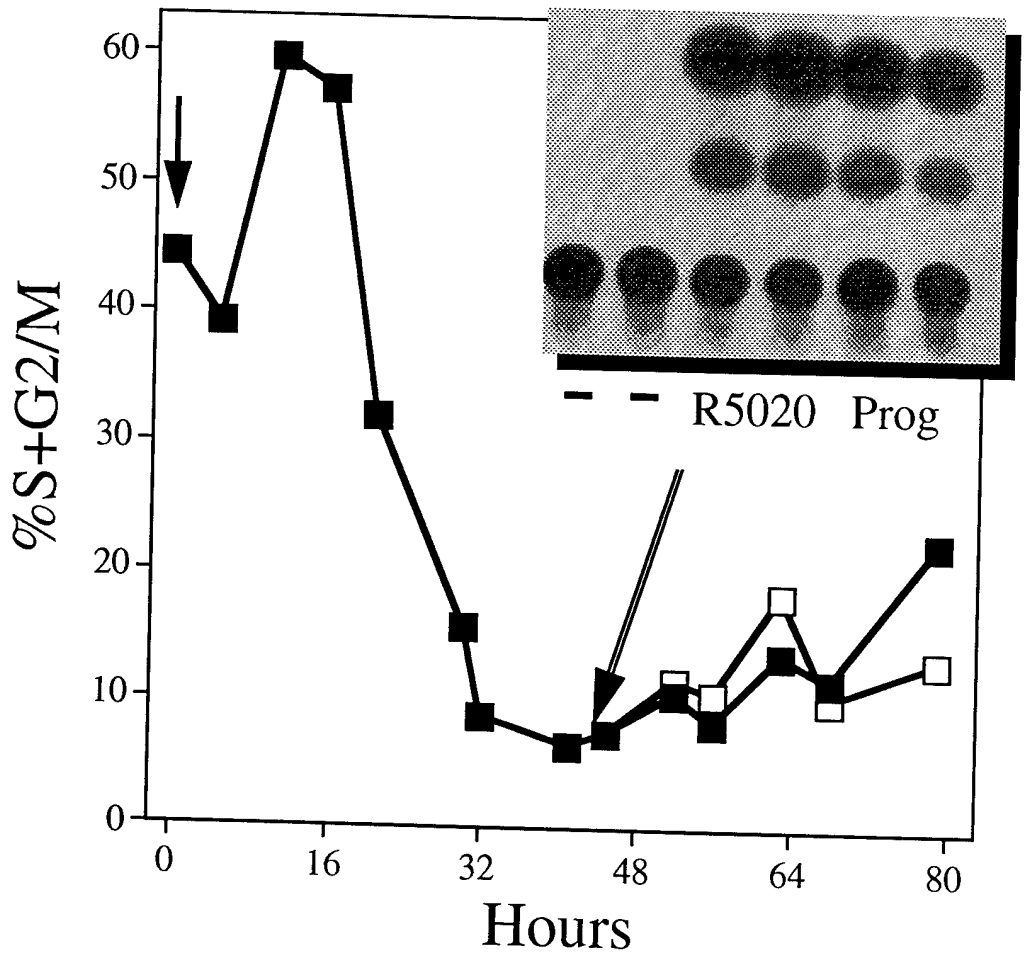
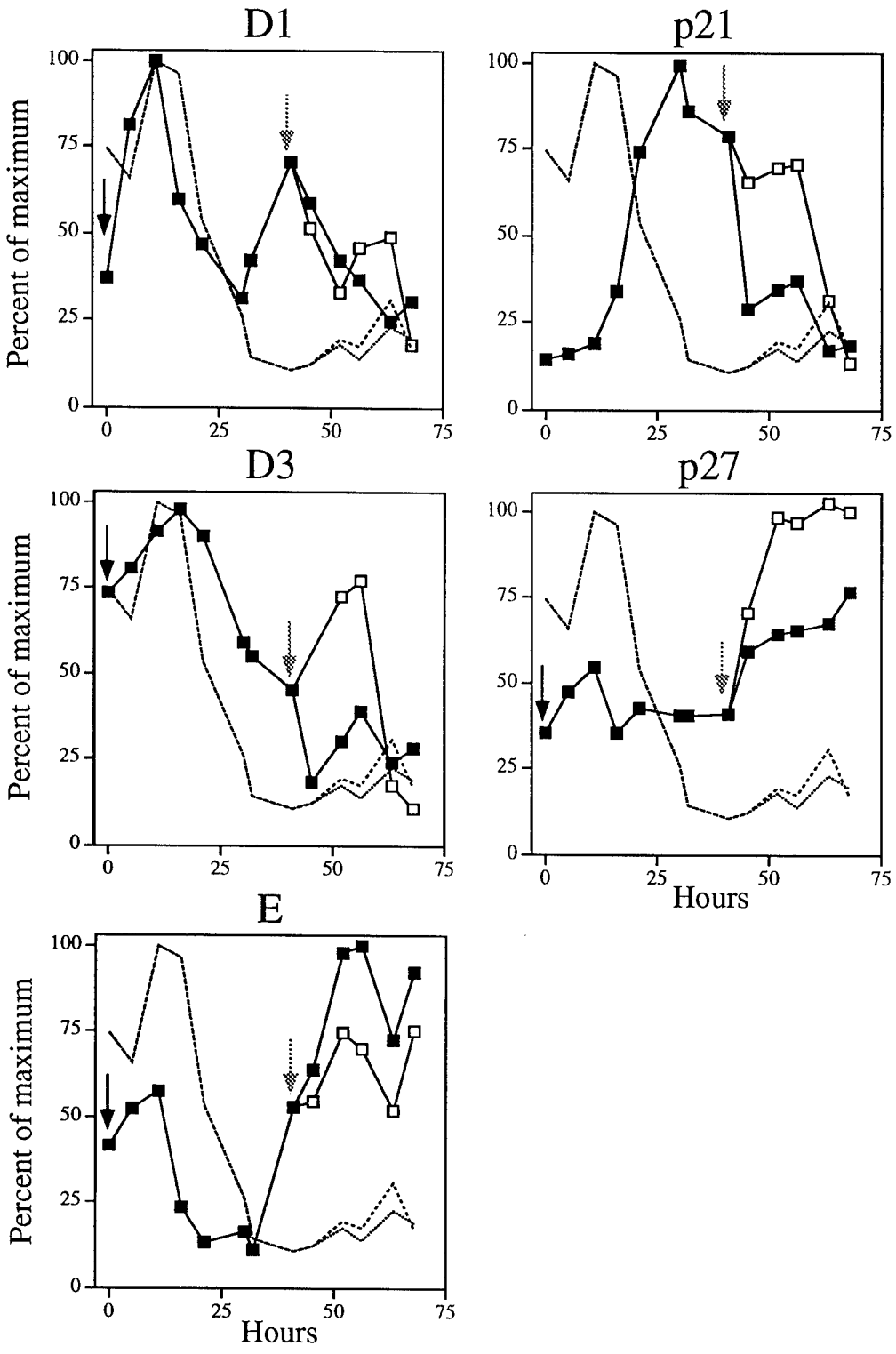

B
R5020
Progesterone
hours 0 10 20 26 32 38 44 50

Figure 3




D1
p21
D3
p27
E
Percent of maximum
Hours
0
25
50
75
100

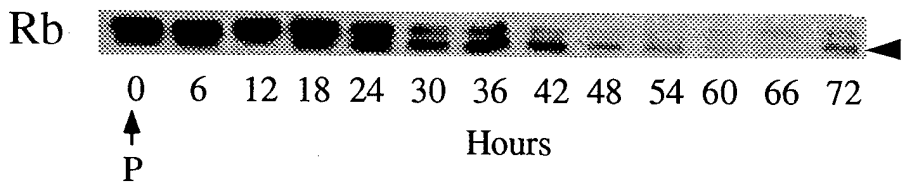
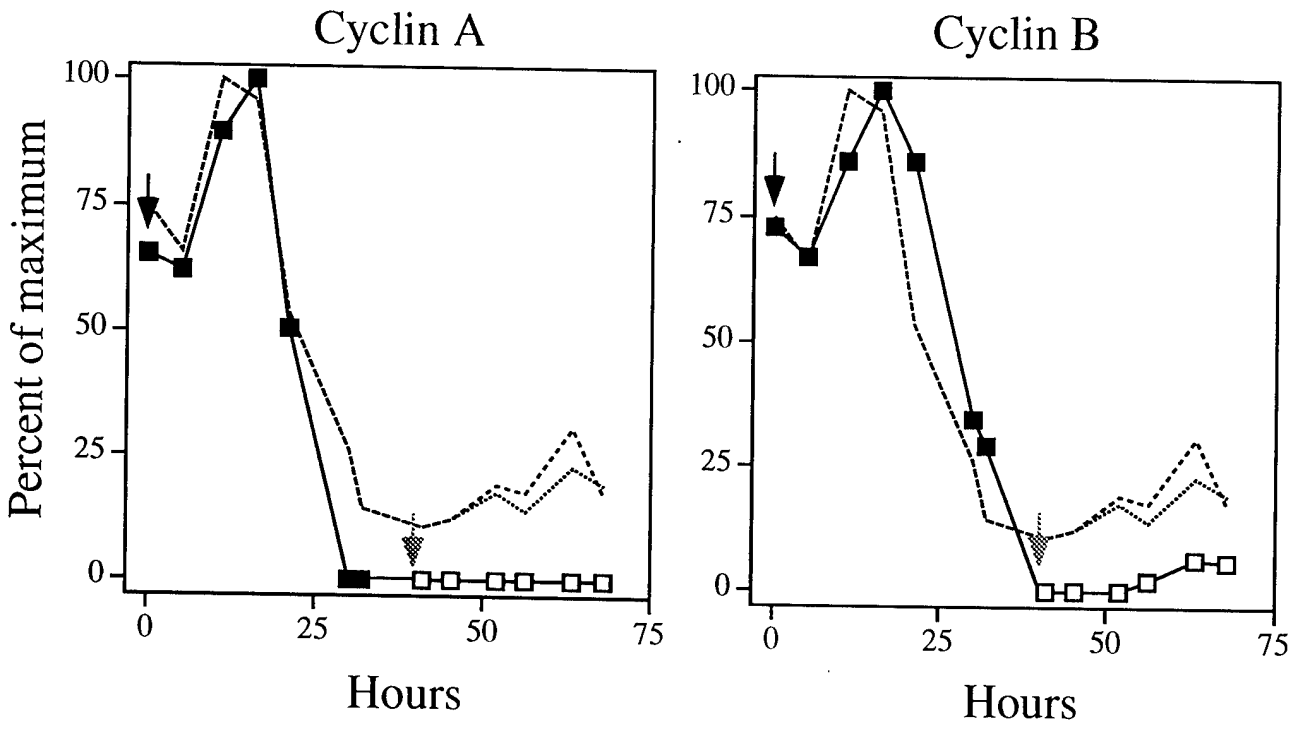
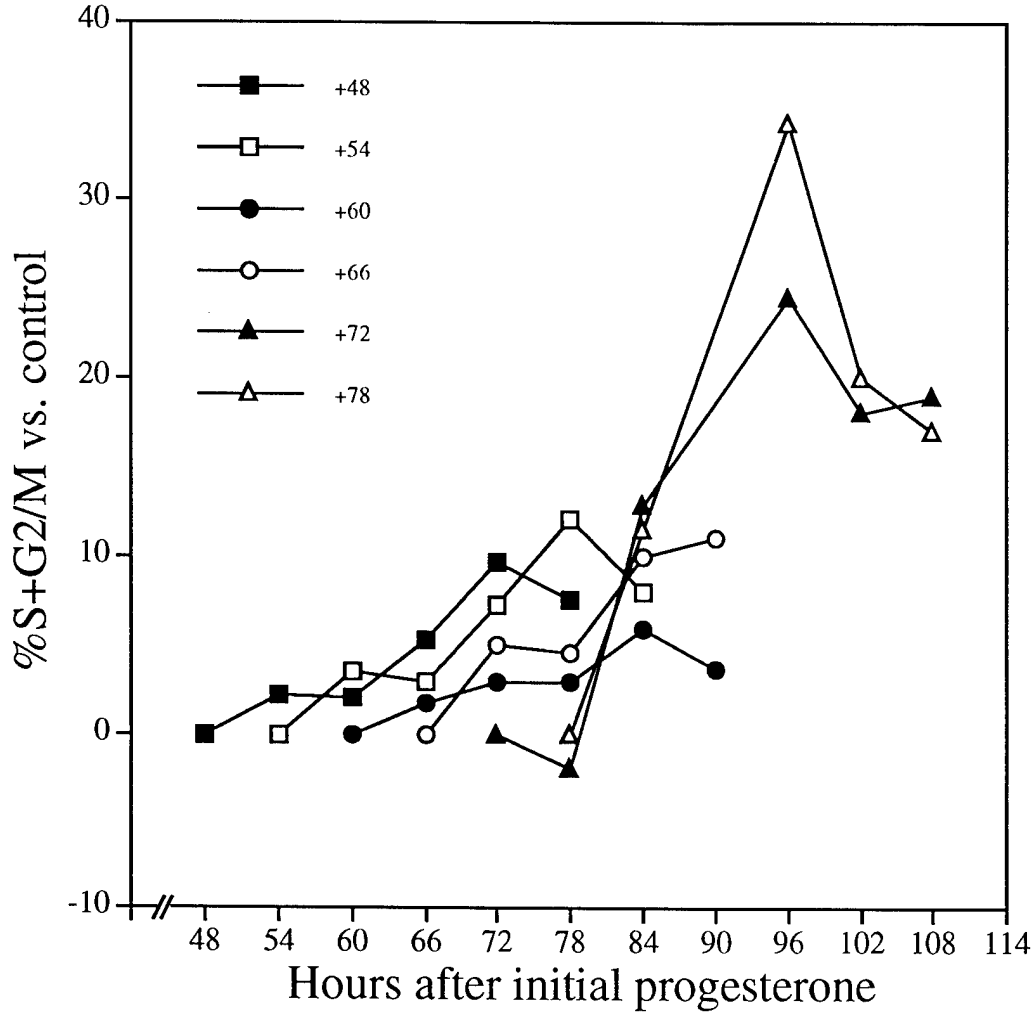

Rb
0 6 12 18 24 30 36 42 48 54 60 66 72
Hours
P

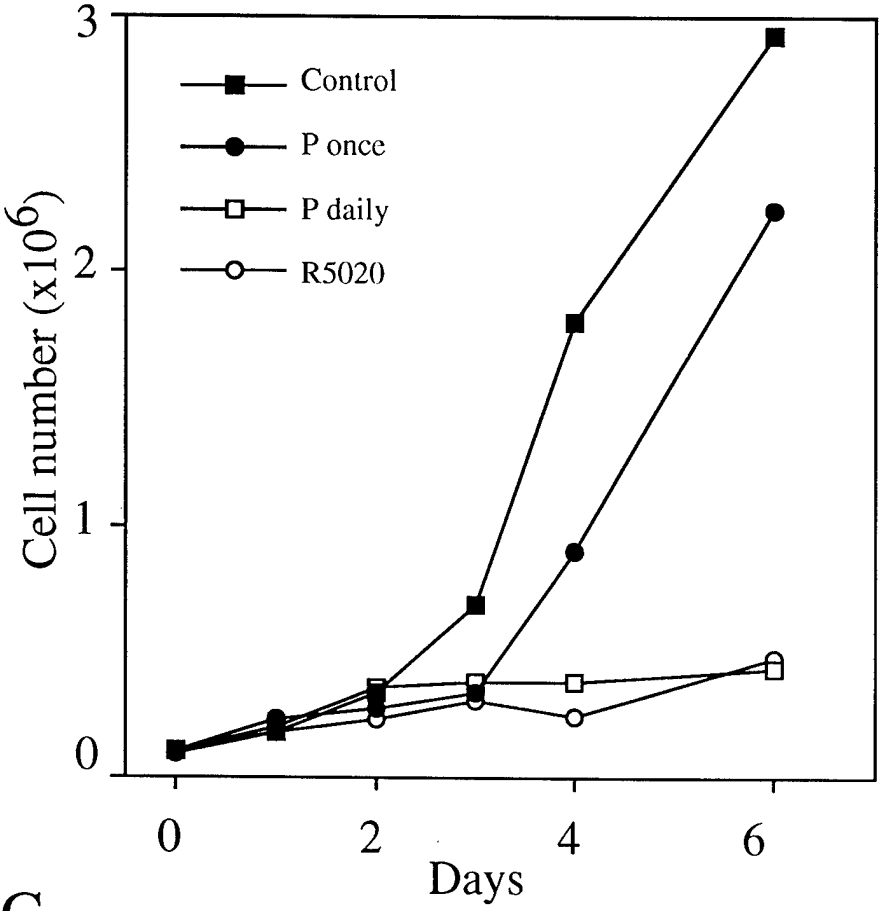
Figure 5


Cyclin A
Cyclin B
Percent of maximum
100
75
50
25
0
0
25
50
75
Hours

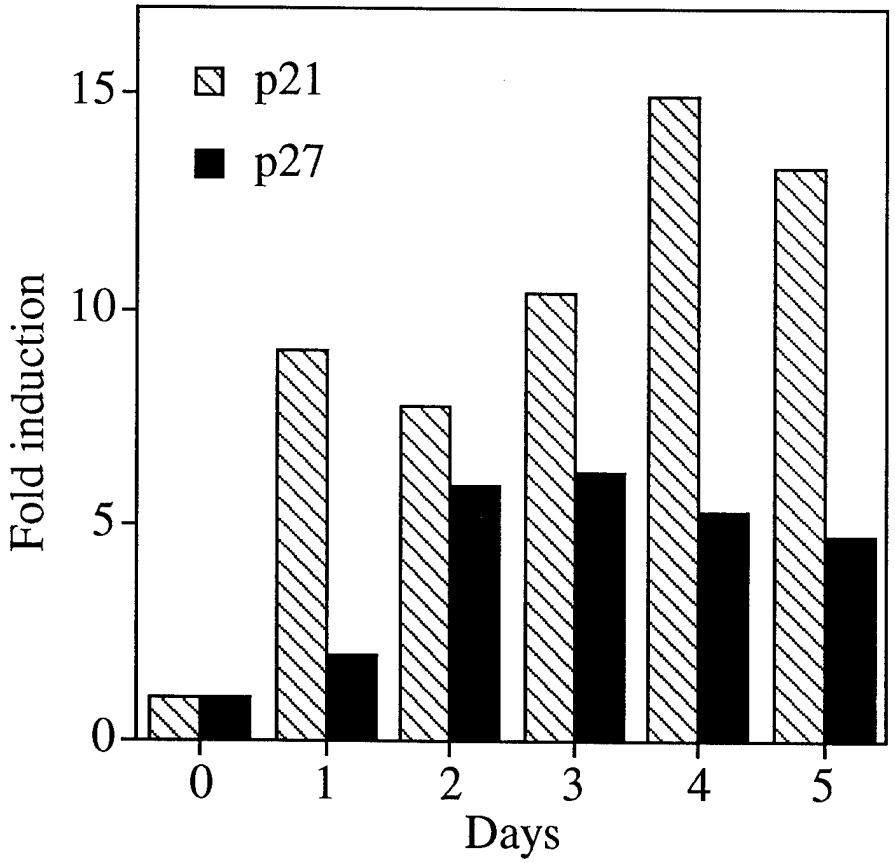
A


40
30
20
10
0
-10
%S+G2/M vs. control
+48
+54
+60
+66
+72
+78
48
54
60
66
72
78
84
90
96
102
108
114
Hours after initial progesterone

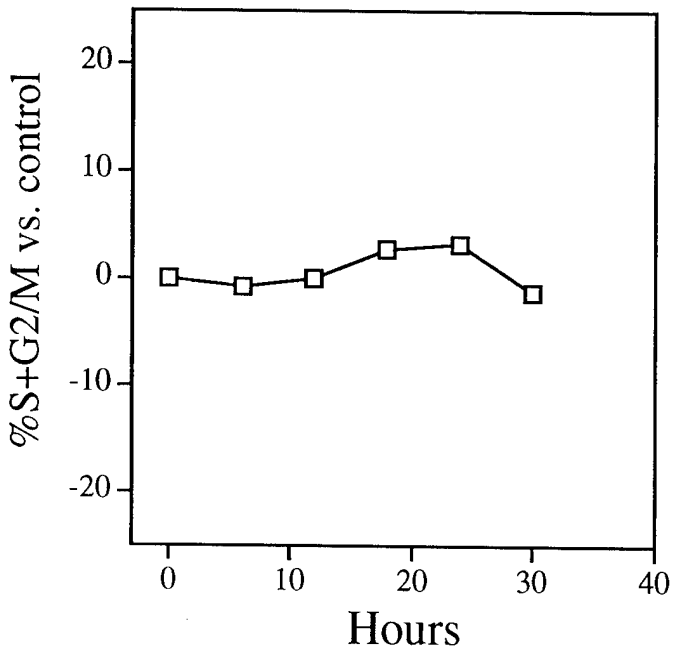
B


Control
P once
P daily
R5020
Cell number (x10^6)
3
2
1
0
0
2
4
6
Days

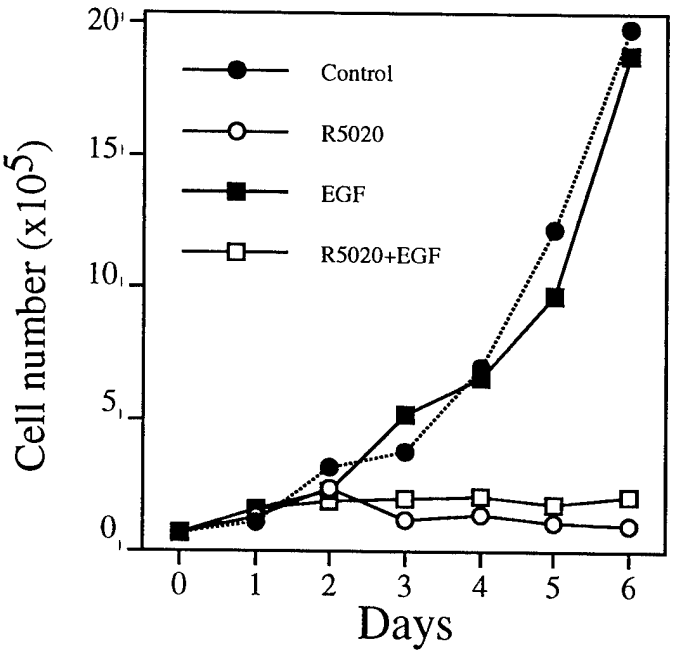
C


p21
p27
Fold induction
15
10
5
0
0
1
2
3
4
5
Days

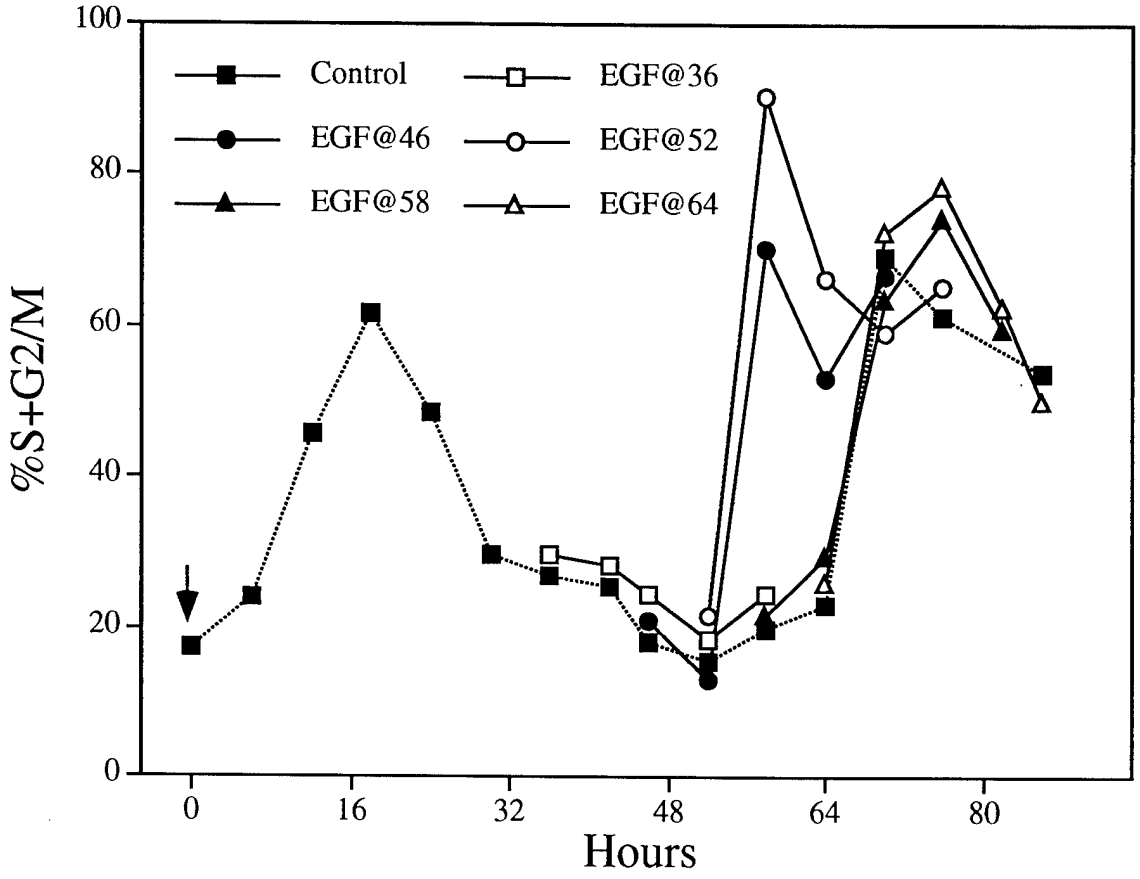
A


%S+G2/M vs. control
20
10
0
-10
-20
0
10
20
30
40
Hours

B


Cell number (x10^5)
20
15
10
5
0
Control
R5020
EGF
R5020+EGF
0
1
2
3
4
5
6
Days

C


100
80
60
40
20
0
%S+G2/M
Control
EGF@36
EGF@46
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EGF@64
0
16
32
48
64
80
Hours

D

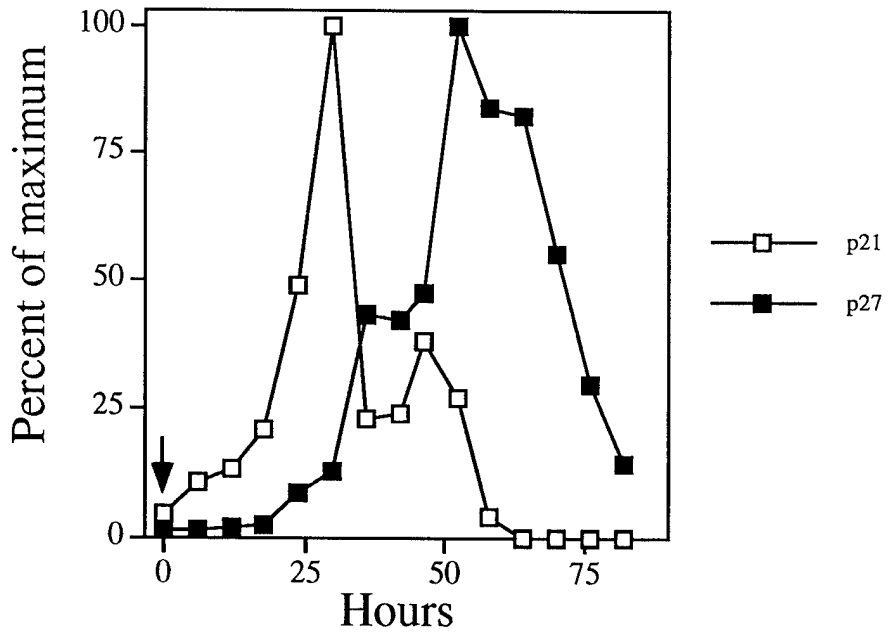
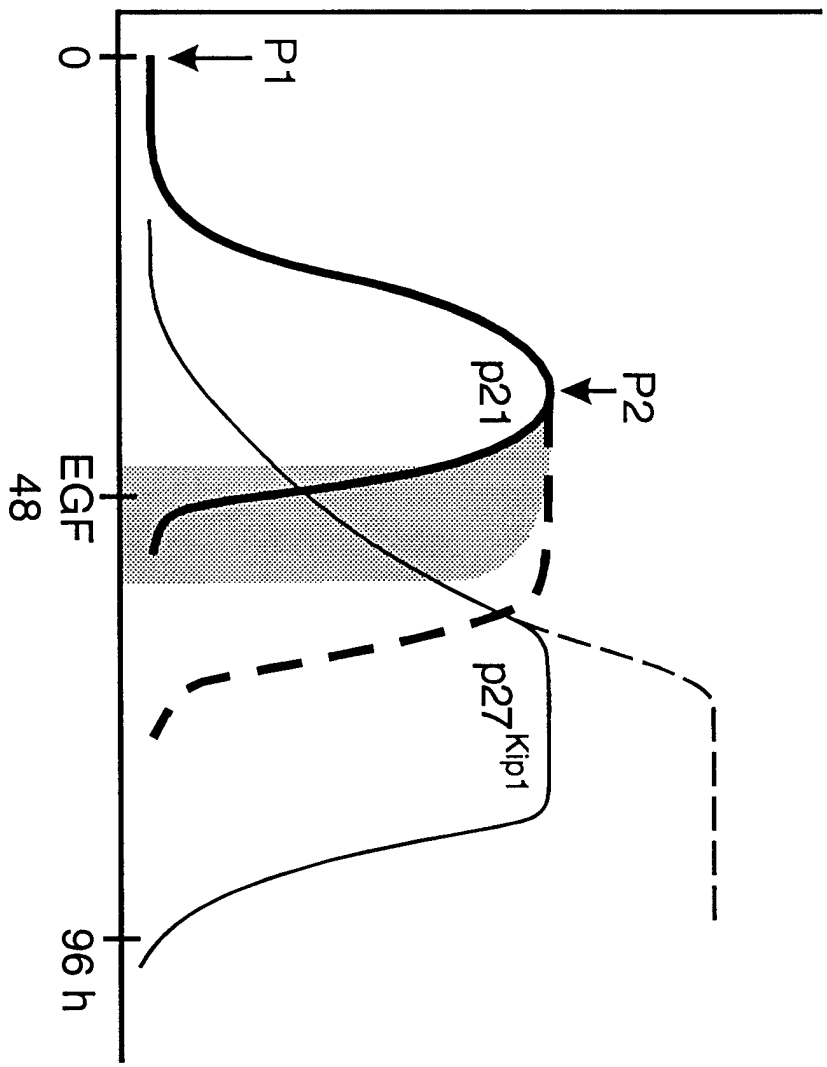

100
75
50
25
0
Percent of maximum
0
25
50
75
Hours
p21
p27

Figure 8



THREE ACTIVATION FUNCTIONS (AFs) AND A NOVEL N-TERMINAL INHIBITORY FUNCTION (IF) IN HUMAN PROGESTERONE RECEPTOR A AND B ISOFORMS CONTROL THEIR TRANSCRIPTIONAL DIVERSITY

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Running Title: Transcription Domains of PR

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ABSTRACT

The B-isoform of human progesterone receptors (PR) contains three activation functions (AF3, AF1, AF2), two of which (AF1, AF2) are shared with the A-receptor isoform. AF3 is in the B-upstream segment (BUS) at the far N-terminal 164 amino acids of B-receptors, AF1 is in the 392 amino acid N-terminal region common to both receptors, and AF2 is in the hormone binding domain. Here we show that cooperativity among these AFs accounts for promoter and cell-specific differences in the transcriptional activity of the two receptors. On two promoters -- the natural promoter of the mouse mammary tumor virus LTR, and a synthetic promoter that contains two progesterone response elements -- B-receptors have five to ten-fold greater transcriptional activity than A-receptors. This is due to transcriptional synergy produced by cooperativity between AF3, and one of the two downstream AFs. Besides cooperative activity, each AF exhibits autonomous transactivating capacity when it is linked alone to the DNA binding domain of PR. The extent of this transcription is, however, promoter- and cell-specific, and is generally higher in HeLa cervicocarcinoma cells, than in T47D breast cancer cells. In addition to transactivating activity, we find that the N-terminus of PR common to both isoforms, contains an inhibitory function (IF), located in a 292 amino acid segment lying between AF3 and AF1. IF suppresses the activity of A-receptors, but is not inhibitory in the context of B-receptors, due perhaps to constraints on its repressor function imparted by BUS. As a result, IF inhibits AF1 or AF2, but not AF3. These data demonstrate the existence of a novel inhibitory function in PR, which, together with the three AFs, accounts in part for the complex transcriptional repertoire of these receptors. Furthermore, mapping of IF to the N-terminus begins to assign novel functions to this large, relatively undefined, structural region of human PR.

INTRODUCTION

Activation domains -- Gene expression in response to extracellular signals is a complex process. It usually involves binding of regulatory protein factors to specific enhancer elements, resulting in transcriptional control of the target gene. These regulatory proteins contain activation domains (AFs) through which they contact the basal transcription machinery either directly, or indirectly by means of intermediary coactivator proteins (1 and references therein). Progesterone receptors (PR) are members of the steroid receptor family of ligand-inducible transcription factors. These are structurally complex nuclear proteins, that contain multiple functional domains, including a highly conserved DNA-binding domain (DBD) composed of two zinc fingers, a moderately well conserved C-terminal hormone-binding domain (HBD), and a highly variable N-terminal region (2,3 and references therein). Two major AFs have been identified in most steroid receptors: in general, one AF (AF1) maps to the N-terminal variable region while the other (AF2) is contained within the HBD. AF1 is constitutively active when the inhibitory HBD is removed, and AF2 requires hormone for activation (4-6).

The AFs of glucocorticoid (GR) and estrogen receptors (ER) are well defined. Two AFs, called *tau*1 (*t*1) and *tau*2 (*t*2), have been mapped by deletion mutagenesis of human (h)GR. They function autonomously when linked to the DBD of the yeast activator GAL4 (7) in the appropriate cellular context (8). *t*1 was originally defined as a 200 amino acid domain in the N-terminus, within which a 41 amino acid core region has recently been mapped (9). *t*2 is 30 amino acids long and is located in the HBD. Although both are highly acidic, they are structurally unrelated, and share little homology with the acidic activation domains (AAD) of GAL4 and GCN4 (7). However, substitution of an acidic amphipathic α-helix for *t*2 produces a transcriptional pattern

similar to that generated by *t*2 (7). Additionally, squelching/interference assays show that hGR *t*1 is strongly inhibited, while *t*2 is weakly inhibited, by co-expression of the viral acidic activator, VP16. These data suggest that the AFs of hGR share a common mechanism of action with the AAD despite the fact that they lack structural resemblance; that the HBD contains multiple or discontinuous AFs; and that *t*1 and *t*2 are functionally distinct and represent different classes of AFs (10).

Two non-acidic AFs in hER are distinct from the AADs, GAL4 and VP16 (10,11). They are capable of autonomous activity on some promoters and in some cell lines, but both AFs are generally required for maximal activity (10). AF2 of mouse (m)ER maps to a 60 amino acid region in the HBD, within which, a 14 amino acid domain (amino acids 538 to 552), contains three critical residues that are essential for activity (12). This domain is conserved among members of the nuclear receptor family, and mutations of the corresponding critical residues in hGR abolishes transcriptional activity (12). These sites, however, are distinct from *t*2, which also supports the hypothesis that transcriptional activation, at least by AF2, is conferred by a discontinuous domain (10).

There are two naturally-occurring, transcriptionally active isoforms of hPR -- the A- and B-receptors. B-receptors are 933 amino acids in length and contain an N-terminal 164 amino acid upstream segment (BUS) that the 769 amino acid A-receptors lack (13-15). The two hPR isoforms differ functionally, depending on the cell, the promoter, and the ligand tested (8,16-18). Additionally, A-receptors are capable of acting as *trans*-dominant inhibitors of B-receptors (17,19,20) and of other members of the steroid receptor family (21). In contrast, only B-

receptors inappropriately activate transcription when they are occupied by antiprogestins (17,22,23). The two hPR isoforms have AF1 and AF2 in common (18,24,25). AF2 is located within the HBD and has not been characterized. AF1 maps to a 91 amino acid, "proline-rich" segment, located just upstream of the DBD. It has no significant homology to other "proline-rich" activation domains (24). Isoform-specific functional differences have been attributed, in part, to a discontinuous third AF (AF3) located primarily in BUS, but which is dependent on the presence of the PR DBD for its activity (18). Unlike AF1 and AF2, BUS fused to a heterologous DBD, such as that of ER (18) or GAL4 (24), is inactive. AF3 may therefore represent a third class of AFs that is distinct from the other two, and has to date been defined only for hPR.

Cooperative interactions between two AFs is suggested by the fact that while on some promoters and in some cell lines each AF is autonomously active, in general, the presence of both AFs is required for full activity (7,8,10,11). In settings where the individual AFs are only weakly active, this cooperativity leads to transcriptional synergism (10,11,18). Tora et al (11) demonstrated cooperativity between AFs of ER even when they are on separate molecules. When either AF1 or AF2 is fused to the GAL4-DBD and co-expressed with truncated forms of hER containing the complementary AF, each AF synergizes with itself and with the other AF (11). Synergism can even occur between the AFs of different receptors -- hGR AF2 synergizes with hER AF1 and/or AF2 (10).

Since hPR have three AFs, study of their autonomous, and cooperative actions is more complex. We have constructed a series of PR fragments in which each region known to contain an AF was linked to the DBD of PR, either alone or in combination with each other AF. In this

paper we describe the autonomous and cooperative transcriptional characteristics of each AF on two different promoters and in two different cell lines.

Inhibitory domains -- In addition to activation functions, recent evidence indicates that transcription factors also contain inhibitory domains that serve to modulate the activity of the AFs. For example, the yeast transcriptional activator ADR1 contains at least three AFs plus a zinc finger-structured DBD. Additionally, there are two regions which, when removed, increase ADR1 activity (26). One of the inhibitory regions represses the activity of a heterologous bacterial transactivator when fused to it, suggesting that a repressor mediates inhibition (26). Similarly, PHO4 is a basic helix-loop-helix transcription factor whose activity is inhibited by the repressor, PHO80 (27). Two regions distinct from the AFs or the DNA-binding/dimerization domains of PHO4 interact with PHO80. These regions are also required for phosphate-mediated repression of PHO4 when it is fused to the heterologous transcriptional activator, LEX A (27). Finally, the N-terminus of c-Fos contains an inhibitory domain (ID1) that silences the activities of one of its activation domains. Mutagenesis of residues thought to constitute an inhibitor motif within ID1 enhances the transactivation of an AP1-bearing promoter by c-Fos (28).

PR A-receptors have two interesting inhibitory functions that B-receptors lack. We (18) and others (8,19) have demonstrated that A-receptor-mediated transcription decreases as its concentration increases; an effect known as self-squelching. Squelching has been defined as the sequestering of limiting coactivators from the transcription complex by overabundance of a coactivator-binding protein, resulting in a bell-shaped transactivation curve (29). Self-squelching is not observed for B-receptors over a wide range of concentrations, suggesting that BUS alters the

conformation of B-receptors so that the squelched factor fails to bind, or that BUS binds a cofactor unique to B-receptors that neutralizes the squelched factor or overrides dependency on it.

Additionally, we have shown that under conditions in which B-receptors are strong transactivators, and transcription by A-receptors is weak, absent or inhibitory, the inhibitory phenotype of A-receptors is dominant over the stimulatory phenotype of B-receptors in the A/B heterodimer (20). This dominant, A-receptor-mediated repression extends even to other members of the steroid receptor family, including ER (19,21).

Because A-receptors are dominant negative inhibitors of B-receptors, and only A-receptors self-squelch, we postulated that A-receptors contain inhibitory sequences distinct from the three defined AFs, and that these sequences are masked in B-receptors. We sought this inhibitory function in a previously uncharacterized 292 amino acid region (IF) of the A-receptor N-terminus. In these studies, the activity of several constructs that either contain or lack IF were compared. We show evidence that IF has a novel repressor function. Specifically, we demonstrate that removal of IF converts A-receptors from weak into strong transactivators, equivalent to B-receptors.

MATERIALS AND METHODS

Recombinant Plasmids -- Complementary DNAs, hPR2 and hPR1, encoding A- and B-receptors, respectively, cloned into the pSG5 expression vector (15) were gifts from P. Chambon (Strasbourg, France). Construction of BUS-DBD, N-terminal B (NT_B)-DBD, N-terminal A (NT_A)-DBD, DBD-HBD and BUS-DBD-HBD expression vectors, all containing a nuclear localization signal (NLS), was described in Sartorius et al (18). NT_A-ΔIF was made by polymerase chain reaction (PCR) amplification of hPR sequences encoding AF1, DBD, and NLS (amino acids 456-644). The upstream PCR primer contained an *Eco*R1 site, Kozak consensus sequence (30) and an ATG initiation codon. The downstream primer contained a STOP codon and *Bgl*II site (18). The resulting PCR fragment was cloned into pSG5 digested with *EcoRI/Bgl*II. A-ΔIF was made by digesting NT_A-ΔIF with *Bbs*I/*Bgl*II and isolating the larger fragment which contains AF1 and the N-terminal portion of the DBD upstream of the *Bbs*I site. This was used as a recipient for ligation of the *Bbs*I/*Bgl*II fragment from BUS-DBD-HBD which contains the C-terminal portion of the DBD and the HBD. A-ΔAF1 was constructed from B-ΔAF1. For the latter, fragment 1, encoding regions upstream of AF1, was made by amplification of hPR1 sequences using a 5' sense primer (nt 1855 to nt 1878, ref. 13) containing an *Mlu*I site, and a 3' antisense primer (nt 2096 to nt 2116) containing a *Sal*I site. Fragment 2, which spans receptor sequences from the 3' border of AF1 to the end of the HBD, was made with a 5' sense primer (nt 2385 to nt 2404) containing a *Sal*I site, and a 3' antisense primer (nt 3525 to nt 3545) containing a *Bgl*II site (18). The two fragments were combined with an *MluI/Bgl*II vector-containing (fragment 3), derived from hPR1. All three fragments were ligated to produce B-ΔAF1. A-ΔAF1 was made from B-ΔAF1 by removing an *EcoRI/Bam* HI fragment, filling in overhanging ends, and self-ligating the blunt ends.

For NT_B-ΔAF1, fragment 4, spanning sequences from the 3' end of AF1 through the NLS, was amplified from NT_B-DBD using the fragment 2 5' sense primer containing a *SalI* site, and a 3' antisense primer (nt 2646 to nt 2678) containing a *BglII* site, STOP codon and coding sequences homologous to the 3' end of NT_B-DBD (18). PCR fragments 1 and 4 were combined with the *MluI/BglII* vector fragment 3 from hPR1 and ligated. The sequence of the cDNAs and expression of the proteins was verified by dideoxy sequencing (U.S. Biochemical Corp., Cleveland, OH) and immunoblotting.

The mouse mammary tumor virus (MMTV)- chloramphenicol acetyl transferase (CAT) and thymidine kinase (*tk*)-CAT reporters were gifts from P. Chambon (15). For PRE_2-$TATA_{tk}$-CAT, two copies of the tyrosine amino transferase (TAT) progesterone response element (PRE; 31) were cloned upstream of a truncated fragment (-60/+51) of the *tk* gene (32,33) as previously described (17).

Immunoblotting -- Whole-cell 0.5 M KCl extracts were prepared from COS cells transiently transfected with each of the expression vectors described. The expressed hPR fragments were separated by electrophoresis on a 7.5% or 11.5% sodium dodecyl sulfate (SDS) denaturing polyacrylamide gel and transferred to nitrocellulose. Protein blots were probed with a mixture of our anti-hPR monoclonal antibodies, AB-52 and B-30 (34) and/or with the anti-DBD polyclonal antibody α266 (35) provided by D. Toft (Rochester, MN). The receptor bands were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) as previously described (23,36).

Transfections and Reporter Assays -- HeLa cells were plated in 100 mm tissue culture dishes in 10 ml Minimum Essential Medium supplemented with 5% twice charcoal-stripped, heat-inacti-

vated fetal calf serum (DCC-MEM) approximately 24 hrs prior to transfection. Cells were transfected by calcium phosphate precipitation (37) with 2 μg of the reporter plasmid, variable amounts of the expression vectors (indicated in the figures), 3 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia-LKB Biotechnology, Piscataway, NJ) to correct for transfection efficiency, and Bluescribe (Stratagene, La Jolla, CA) carrier plasmid for a total of 20 μg/plate. Twenty-four hrs after transfection, the medium was changed to 7.5% DCC-MEM, and cells were either left untreated or were incubated with 10 nM of the synthetic progestin, R5020 (Roussel UCLAF, France), for an additional 24 hrs. Cells were harvested, lysates were normalized to β-galactosidase activity, and then analyzed for CAT activity by thin layer chromatography (TLC) as previously described (17,22). Standard deviations of phosphorimaging (Molecular Dynamics, Sunnyvale, CA) data were determined using Microsoft Excel Version 5.0 (Microsoft Corporation) for the number of sets indicated in the figure legends.

Transfection of T47D-Y cells was performed as previously described (23). Briefly, cells plated in 100 mm dishes in 5% DCC-MEM were transfected by calcium phosphate precipitation, with 3 μg reporter plasmid, receptor expression vectors at concentrations indicated in legends, 4 μg of pCMV-βgalactosidase expression vector (Clontech, Palo Alto, CA), and carrier plasmid for a total of 20 μg/plate. After 24 hrs, the cells were glycerol shocked as described (23), and the cell medium was changed to 7.5% DCC-MEM. Cells were hormone treated for 72 hrs, harvested and analyzed for CAT activity and statistically analyzed as described above.

RESULTS

Autonomous Activity of Each AF -- To study the transcriptional activity of the three AFs, a series of expression vectors were constructed in which each region of hPR that contains an AF was fused, either alone or in combination with another AF, to the DBD of hPR. All constructs also contain the endogenous hPR nuclear localization signals (NLS) (38). Figure 1 shows their names and structures. The structure of four constructs used to test the function of the far N-terminus (aa 165-456) of A-receptors lying upstream of AF1, which we have designated IF, is also shown.

Expression of PR protein fragments of the expected size was measured by immunoblotting following transient transfection of 1.25 μg per 100 mm plate of the appropriate expression vectors into COS cells, as shown in Figure 2. COS cells were used as the recipients since they express the large T-antigen required to transiently produce peptides from pSG5 at the high copy number optimal for immunoblotting. This computer-generated figure demonstrates the approximate migration distance, using either 7.5% or 11.5% acrylamide, of the non-hormone treated receptor fragments used in these studies, which range in size from 191 to 933 amino acids. They are all well expressed, although there is some apparent variability in their levels. Much of this is due to the effects of phosphorylation, which produces peptide fragments that migrate as multiple bands. We have shown that the triplet structure of B-receptors (lane 1) is due to phosphorylation of several serine residues located on BUS (18,39 and unpublished data). For this reason many of the fragments that contain BUS -- BUS-DBD-HBD (lane 4); NT_B-DBD (lane 6); NT_B-ΔAF1 (lane 7), BUS-DBD (lane 10) -- also contain multiple bands. Through unknown mechanisms, this multiple banding pattern is amplified by removal of the HBD. Additionally, as we have shown (23,40), when full-length B-receptors are overexpressed, low levels (0-5%) of A-receptors are translated

at an internal methionine codon present in the hPR1 construct. These are seen as the lower Mr singlet band in lane 1, that co-migrates with A-receptors (lane 2).

The transcriptional activity of the PR fragments was tested using two promoter-reporters: PRE_2-$TATA_{tk}$-CAT is a synthetic reporter that has two copies of the palindromic PRE found in the TAT promoter, cloned upstream of the ~60 bp region containing the TATA box of the *tk* promoter; MMTV-CAT is a natural promoter that consists of the -631/+125 bp in the upstream LTR of the MMTV gene, and contains one palindromic PRE and three PRE half-sites (41,42). The two promoter-reporters were tested in two cell lines: PR-negative HeLa cells derived from a human cervical carcinoma, and T47D-Y cells, a PR-negative subline of the constitutively PR-positive $T47D_{co}$ breast cancer cell line (23). In preliminary studies (data not shown) various concentrations of expression vectors encoding the PR constructs, were cotransfected into cells together with reporters, to determine the concentrations that yield maximum transcriptional activity. In general, these concentrations, the details of which are supplied in the figure legends, were chosen for the studies described below.

Figure 3, panels A-D, shows the CAT activity in both cell lines as measured by TLC, after transient cotransfection of each promoter-reporter with expression vectors encoding wild-type B-receptors, A-receptors, and the PR constructs that contain each AF singly linked to the DBD. Cells expressing PR constructs that contain an HBD were tested in the absence or presence of 10 nM R5020. The empty expression vector pSG5 produces little or no CAT activity from either promoter or cell line whether or not R5020 is present (panels A, B, D, lanes 1-4, and data not shown).

B-receptors, which contain all three AFs, are inactive in the absence of hormone (panels A-D, lanes 5, 6) but are strong CAT transactivators in the presence of R5020 on both promoters, and in both cell lines (panels A-D, lanes 7, 8). In the quantitative analyses shown in figure 5, the activity of B-receptors has been set at 100%, and the activity of the other receptor constructs is measured relative to that of B-receptors. Fold-induction over baseline is not assessed, since basal levels are at or near zero.

In contrast to B-receptors, A-receptors, which lack AF3, are weak hormone-inducible transactivators in both cell lines and on both promoters (Figure 3, lanes 11, 12). In HeLa cells (panels A, B), A-receptors have approximately 10% the activity of B-receptors (compare lanes 7, 8 and 11, 12). In T47D-Y cells, A-receptors are also weak transactivators, having little or no activity when PRE_2-$TATA_{tk}$ is the test promoter (panel C, lanes 11, 12), and only modest activity (~20% that of B-receptors) on the MMTV promoter (panel D, lanes 11, 12).

The remaining constructs in figure 3 each contain a single AF linked to DBD. NT_A-DBD, the N-terminus of A-receptors, contains AF1; DBD-HBD contains AF2; and BUS-DBD contains AF3. The autonomous activity of these AFs shows strong promoter specificity in HeLa cells -- in general, they are much more active on PRE_2-$TATA_{tk}$ (panel A) than on MMTV (panel B). Thus, compared again to B-receptors, AF1 (NT_A-DBD) is approximately 15% active on PRE_2-$TATA_{tk}$ at the concentrations tested, but has little apparent activity on MMTV (lanes 13, 14); AF2 (DBD-HBD) is 70% active on PRE_2-$TATA_{tk}$ and 30% on MMTV (lanes 17, 18); and most discordant, AF3 (BUS-DBD) is 90% active on PRE_2-$TATA_{tk}$ and 5% on MMTV (lanes 19, 20). In T47D-Y cells all the individual AFs have low activity. Cell specific differences are particularly evident with

PRE_2-$TATA_{tk}$-CAT on which autonomous AF activity is strong in HeLa cells (panel A, lanes 13, 14; 17, 18; 19, 20) but absent in T47D-Y cells (panel C).

The data using HeLa cells illustrate an interesting observation with respect to A-receptors -- namely that the individual AFs, AF1 and AF2, have stronger autonomous activity when each is present alone, than when they are combined in the full-length A-receptors. This is particularly evident on PRE_2-$TATA_{tk}$ (panel A), and suggests that on some promoters, an inhibitory activity is associated with full-length A-receptors. This is not the case with B-receptors as is described below.

Cooperativity Among AFs -- To study the interactive behavior among AFs -- either to suppress or to enhance transcription -- two additional PR constructs were analyzed: BUS-DBD-HBD, which contains AF3 and AF2, and NT_B-DBD, the N-terminus of B-receptors, which contains AF3 and AF1. Recall that A-receptors represent the combination of AF1 and AF2, and B-receptors represent the sum of all three AFs. The new constructs were again tested on PRE_2-$TATA_{tk}$-CAT and MMTV-CAT, in both HeLa and T47D-Y cells and were compared to B-receptors, as shown in figure 4. Data on A-receptors are found in Figure 3.

The studies in Figure 4 illustrate important promoter-specific differences in the combinatorial effects of the AFs of PR, which are influenced by cell context. First, following hormone treatment, the combination of AF3 and AF2 (BUS-DBD-HBD) has activity equal to that of B-receptors on both promoters and in both cells (Figure 4, panels A-D; compare lanes 7, 8 with 3, 4). This is true whether the activity of each AF alone is strong, as it is in HeLa cells on PRE_2-$TATA_{tk}$ (Figure 3, panel A, lanes 17-20), or whether the activity of each AF alone is weak, as it is

in T47D-Y cells on PRE_2-$TATA_{tk}$ (Figure 3, panel C, lanes 17-20). However, because of the weak activity of each AF in T47D-Y cells, the combination of AF3 and AF2 is synergistic, since transcription is much greater with both (95% compared to B-receptors), than it is with each of the component AFs alone (no more than 2 or 3% for each). A synergistic effect cannot be demonstrated in HeLa cells, where these two AFs are potent transactivators alone.

The combination of AF3 and AF1 (NT_B-DBD) demonstrates important promoter differences (Figure 4, lanes 9, 10). Their combined activities equal that of B-receptors (set at 100%) on PRE_2-$TATA_{tk}$-CAT in HeLa cells (panel A), but are less than 10% of B-receptors on MMTV-CAT in the same cells (panel B). Similarly, in T47D-Y cells, the combined activity of AF3 and AF1 is more than 90% on PRE_2-$TATA_{tk}$ (panel C) but only 10% on MMTV (panel D). In both cell types, when individual AF activity is low, their combined activity is synergistic; much greater than the sum of the activities of each AF alone.

Data using all the constructs, analyzed on both promoters and in both cells, are quantitated and summarized in Figure 5. Where possible, each bar represents the average of multiple experiments (as detailed in the figure legend) and shows the statistical range of variability for transcription by each construct. Results from HeLa cells are shown on top, T47D-Y cells on the bottom, PRE_2-$TATA_{tk}$ in panels A and C, and MMTV in panels B and D. In all four sets, the activity of B-receptors far exceeds that of A-receptors (AF1 and AF2). When comparing PRE_2-$TATA_{tk}$ in the two cells, the individual AFs have much higher relative activity in HeLa cells than they do in T47D-Y cells (compare panels A vs. C). This difference, when using PRE_2-$TATA_{tk}$, is eliminated with constructs containing AF3 and AF2, or AF3 and AF1, whose activity equals that of B-

receptors in both cells. Taken together with the fact that the combination of AF1 and AF2 is uniformly low with this reporter in both cells, our data suggest that in these models, AF3, which is unique to B-receptors, is the primary contributor to transcriptional synergy. In contrast, the combination of AF1 and AF2 in A-receptors is inhibitory; an effect that is especially evident under conditions in which each of these AFs alone is strong (panel A).

Figure 5 also demonstrates that the transcriptional synergy between AF1 and AF3 is promoter-specific. The combination of AF3 and AF1 is strong (100% of B) on PRE_2-$TATA_{tk}$ (panel A) but weak (less than 10% of B) on MMTV (panel B), in both HeLa and T47D-Y cells (compare AF3/AF1, in panels A,C and B,D). Considering the structural differences between the two promoters, it is possible that the combination of AF3 and AF1 creates a surface that promotes cooperativity between the two PRE palindromes of PRE_2-$TATA_{tk}$; a property that cannot be exploited on the MMTV promoter. If so, in wild-type PR, only B-receptors would have this property. Related to this may be the observation that, in HeLa cells, AF3 is strong (90% of B) on PRE_2-$TATA_{tk}$ (panel A), but weak (3% of B) on MMTV (panel B). However this strong effect of AF3 is not observed in T47D-Y cells, suggesting that factors other than, or in addition to, the organization of the PREs are required for cooperativity.

An Inhibitory Domain? -- The transcription studies described in the preceding section demonstrate the effects of various receptor constructs at a single DNA concentration for the PR expression vectors. In general, the concentration chosen gave maximum transcriptional activity in the test system described. However, for one of these constructs, NT_A-DBD, which is the N-terminus of A-receptors, an optimum DNA concentration was difficult to choose, since, as shown in Figure

6, its transcriptional activity increased in direct proportion to the DNA concentration. Data using PRE_2-$TATA_{tk}$-CAT transcription in HeLa cells by several concentrations of full-length B- and A-receptors, and by NT_A-DBD are shown in this study. NT_A-DBD was of interest since it lacked only the HBD of A-receptors, yet it differed from A-receptors in two important respects: it did not "self-squelch", and it had high transcriptional activity. As we have shown (Figures 3-5), in these test systems A-receptors have low transcriptional activity at all DNA concentrations, and additionally, once peak activity is attained, further increases in DNA consistently produce a decrease in transcription (Fig. 6). This "self-squelching" behavior of A-receptors has previously been described by us (18) and others (8,19). The study in Figure 6 shows that NT_A-DBD reach high levels of transcriptional activity. They also do not self-squelch, which suggests that the HBD is, at least in part, responsible for this property. However, if presence of the HBD accounts for self-squelching, then B-receptors which also contain this domain, should also self-squelch? Yet they do not (figure 6). To explain this paradox, we postulated that other receptor regions cooperate with the HBD to produce self-squelching, and focused our studies on a 292 amino acid region of unknown function common to the two receptor isoforms (aa 165 to 455) located between AF3 in BUS (aa 1 to 164) and AF1 (aa 456 to 546). This intervening fragment (IF) could, in theory, have different functional properties in the context of A- vs. B-receptors, since in the former it is at the free N-terminus of the protein, and in the latter it is constrained by BUS.

We therefore compared the self-squelching behavior of full-length A-receptors, with that of A-receptors lacking the IF domain (A-ΔIF) as shown in Figure 7. Since A-ΔIF retains AF1 and AF2, we expected its maximum transcriptional activity to resemble the low levels observed with full-length A-receptors. Surprisingly, on the PRE_2-$TATA_{tk}$ promoter in HeLa cells, deletion of IF

produced an A-receptor whose maximum transcriptional activity was as high as that of B-receptors, particularly at low input DNA concentrations (10 or 100 ng) of the expression vector. However, unlike B-receptors, but like A-receptors, this construct displays strong self-squelching. As the cDNA concentration was increased above 100 ng, the transcriptional activity dropped precipitously to just above basal levels. We conclude that while IF is not required for self-squelching, it carries a powerful repressor domain, which, when removed, converts A-receptors from weak into strong transactivators. The self-squelching behavior was retained due to presence of the HBD unconstrained by BUS (Tung *et al.*, manuscript in preparation).

The strong transcription by A-ΔIF is not, however, due to enhanced cooperativity between AF1 and AF2, caused by removal of IF. This is shown in Figure 8, using constructs in which the activity of each AF alone is tested in the presence or absence of IF under conditions in which cooperativity is precluded. Dose-response data for constructs expressing AF1 (NT_A-DBD) with and without IF are in panel A, AF2 (DBD-HBD) with and without IF are in panel B, and AF3 (BUS-DBD) with and without IF are in panel C. Analogous to its role in full-length A-receptors containing both AFs (Figure 7), with AF1 and AF2 alone, IF has its strongest effects at low receptor concentrations. Thus, at 10 and 100 ng of cDNA, IF suppresses either AF1 or AF2 independently, and removal of IF enhances the transcriptional activity of each AF. These data suggest that IF suppression of AF1 and AF2 is additive in the full-length A-receptors. At higher cDNA concentrations the influences associated with self-squelching and the HBD dominate so that even in the inhibitory presence of IF, transcriptional activity by AF1 without the HBD (panel A) equals the high levels of B-receptors, while even in the stimulatory absence of IF, the HBD suppresses AF2 activity (panel B). Thus there are two independent inhibitory influences in A-receptors:

that of IF at low receptor concentrations; that of self-squelching at high receptor concentrations. Neither of these operates in B-receptors (Figure 8, panel C). Removal of IF does not enhance AF3 activity at low (10 ng) cDNA concentrations, and the HBD does not produce self-squelching of full-length B-receptors at high (1000 ng) cDNA concentrations. Apparently, BUS neutralizes both inhibitory functions.

We conclude that the two complex PR isoforms are assembled from multiple functional domains, among which is an inhibitory domain, and that interactions among these domains can lead not only to transcriptional synergism, but also to silencing.

DISCUSSION

This paper contains a detailed characterization of three AFs and an inhibitory function present in hPR, using two promoters and two human cell lines. We show that the three AFs exhibit promoter and cell-specific autonomous behavior; that cell and promoter-specific positive cooperativity leading to synergistic responses occurs between AF3 and one or the other downstream AF; and that a novel inhibitory function, IF, lies in the 292 amino acid N-terminal region upstream of AF1, that operates only in the context of A-receptors.

A- vs. B-Receptors -- Why progesterone target tissues contain two receptor isoforms remains an intriguing physiological puzzle. Two forms of PR were first described in chick oviducts (43), and then in human cells (13). In humans, the two proteins are the products of a single gene that has two promoters, from which at least nine messages, two A-receptor specific, are transcribed (44). An internal AUG present in some messages may also encode A-receptors (40,44). Thus, there is complex regulatory control over protein levels of the two isoforms, the details of which are still unclear. In initial studies using breast cancer cell lines the two isoforms were found in approximately equimolar amounts (13). However, it is likely that in different tissues, their levels are under tight developmental and hormonal control. The oviduct PR of hens are seasonally and hormonally regulated: A-receptors are lost in winter; B-receptors are lost with aging or following estrogen deprivation (45-48). The two isoforms are developmentally regulated in the brain of the female rat, with the B isoform predominant neonatally, switching to the A isoform pre-pubertally (49). Preliminary data in the human uterus show a similar discordance, with A:B ratios ranging between 50:1 and 2:1 during the menstrual cycle due to large excursions in the levels of B-receptors (50). Graham *et al* (51) analyzed A:B ratios in over 200 PR-positive breast cancers.

They found that equimolarity was the norm, but that a subset of tumors had an excess of A-receptors. Given the functional differences between the two PR isoforms, their unequal distribution among different cells and tissues is likely to have important biological consequences that remain to be explained.

Promoter and Cell Specificity of AFs -- Differential expression of the two PR isoforms provides one level of transcriptional control. Cell and promoter specificity of the autonomous and cooperative behavior among the three AFs provides additional diversity. The activities of the AFs are summarized in Figure 5. Cell type dependence is particularly evident when the autonomous activity of each AF is tested on PRE_2-$TATA_{tk}$-CAT: AF1 (NT_A-DBD), AF2 (DBD-HBD) and AF3 (BUS-DBD) alone are much more active in HeLa cells (Fig. 5, panel A) than they are in T47D-Y cells (Fig. 5, panel C). Promoter dependence is evident in two cases: in HeLa cells the autonomous activity of each AF is much higher on PRE_2-$TATA_{tk}$ (Fig. 5, panel A) than on MMTV (Fig. 5, panel B); and in both cells, cooperativity between AF3 and AF1 is strong on PRE_2-$TATA_{tk}$ (panels A and C) but is weak or absent on MMTV (panels B and D).

Examples abound for cell and promoter specificity of steroid hormone action (8,10,11,16,18,19,22,52), but their underlying mechanisms remain unknown. This is of considerable importance, however, since an understanding of the mechanisms would allow one to target specific organs or genes for desired therapeutic effects on the one hand, and would allow one to minimize unwanted side-effects, on the other. At present, explanations for these differences have focused on target gene structure and on cell-specific coactivator proteins, but these two factors are probably inseparable. The promoters studied herein differ considerably. The two

palindromic PREs on PRE_2-$TATA_{tk}$ are 25 bases apart, and lie 42 bp upstream of the TATA box. An SP1 site from the *tk* promoter is the only known transcription factor binding site (53). MMTV is more complex. Its single palindromic PRE is 200 bp upstream of the TATA box, and an additional three downstream PRE half-sites are separated from the palindrome by at least 55 bases (41,42). This promoter also contains OTF1 and NF1 binding sites (54,55). Thus, differences in the activities of the two promoters could, in theory, be due to PRE distance effects on their ability to interact cooperatively; to the special properties of half-sites; or to involvement of the ancillary transcription factors. Examples of each of these have been described, they have been extensively reviewed (1,3,10,24,42,56 and references therein), and one or all could explain the cell and promoter effects we describe here.

Since these complexities are so difficult to unravel, and apply to transcription in general, recent studies have attempted to define those factors that specifically regulate the activities of steroid receptors. Even more to the point, attempts are now being made to isolate specific coactivators or transcriptional intermediary factors through which different classes of steroid receptor AFs interact with the basal transcription apparatus. AF2 is a particularly attractive target for these studies, since its activity is hormone regulated, providing a physiologically relevant on-off switch for screening candidate proteins. Several proteins that interact with AF2 of ER have recently been described. They include a 160 kDa protein isolated from MCF-7 breast cancer cells (57); proteins of 80, 140 and 160 kDa present in several cell lines (58); the yeast SPT6 protein (59); and $hTAF_{II}30$, an ~30 kDa HeLa cell nuclear protein that co-immunoprecipitates components of the basal TFIID complex including the TATA binding protein (60). TFIIB, another component of the basal transcription complex, interacts directly with several members of the

nuclear receptor family of proteins, including PR (3,61-63). To our knowledge, no N-terminal steroid receptor-specific binding proteins have been described, perhaps because analysis of this receptor region is complicated by its constitutive activity.

Repression of PR Activation Functions -- Much of the work devoted to understanding regulation of transcription by steroid receptors has focused on AFs and their stimulatory actions. However, transcriptional inhibition may be equally important as a way of preventing inappropriate activation, or of terminating hormonal signals and maintaining physiological homeostasis. Studies that deal with transcriptional repression have usually focused on composite DNA elements and invoke mechanisms in which receptor occupancy at one DNA site interferes with transcription by an activator at an adjoining site (64-66 and references therein). Heterodimerization of an activator by a repressor is another silencing mechanism (67). We propose here, as have others (3,26,27,62,63), that both positive and negative signalling elements can reside in the receptor molecule itself; namely, that transcriptional repression can be a function of protein structure rather than DNA structure. We show preliminary data that hPR A-receptors contain a structural element, which we have designated IF, that at least under the conditions described can markedly suppress their transcriptional activity. In this respect, A-receptors differ profoundly from their B-isoform partners. The latter also contain an IF element, but, we argue, its repressor activity is constrained by the tethering influence of BUS located further upstream.

There is now compelling evidence that alterations in the 3-dimensional allosteric structure of steroid receptors modifies their transcriptional behavior. Most of that work comes from analyses of the HBD. For example, using protease accessibility as a probe for receptor structure, it has

been shown that PR (68-70) and ER (71,72) assume altered conformational states when the HBD is occupied by agonist or antagonist hormones. Thus binding of one or another ligand at the HBD conveys structural information that is interpreted as a positive or negative signal by the transcriptional machinery.

More germane with respect to possible effects of BUS and IF on receptor structure, are recent studies which show that the far C-terminal ends of ER and PR impart structural constraints on the molecules. Deletion of a 42 aa region from the extreme C-terminus of the 933 aa PR B-receptors converts antiprogestins from inhibitors to stimulators of transcription. These data suggest that the distal C-terminus of PR contains an inhibitory function that is relieved by hormone treatment (70). The extreme C-terminal "F" domain of ER also appears to function as a inhibitor of activation by hormone (72).

In contrast to these examples, our studies show that IF represses AF1 and AF2 in A-receptors that have already been activated by hormone. We also propose that the proximal N-terminal BUS domain imparts structural information to B-receptors which masks the inhibitory property of IF -- that is, AF3 neutralizes IF. We have previously demonstrated that AF3 transcriptional activity located in BUS is critically dependent on the presence of the hPR DBD. A heterologous DBD does not suffice, so that neither the DBD of the yeast activator GAL4 (24) nor the DBD of ER (18) can reconstitute AF3 activity. The models we propose (18) involve either specific intramolecular contacts between BUS and the DBD, or binding of a bridging protein spanning the two domains. We have some evidence for the latter. In gel mobility shift studies, BUS-DBD binds to a PRE only if nuclear extract proteins or a BUS-specific bivalent monoclonal

antibody are added, suggesting that an accessory binding protein interacts with BUS and the DBD (18).

In sum, we are focusing on the PR N-terminus to explain functional differences between the two isoforms. We suggest models in which the entire B-receptor N-terminus is folded upon itself, or in which B-receptor N-terminal regions are masked by bridging proteins. Either state of B-receptors would strongly influence the structure and accessibility of the internal IF segment, or the availability of IF as an inhibitory surface for protein-protein interactions. These constraints on IF would be removed by deletion of BUS -- a condition that defines A-receptors. As yet, little is known about the 3-dimensional structure of the N-termini of any steroid receptors. This region is, however, structurally the most divergent among members of this family of proteins, suggesting that each receptor will take on unique N-terminal conformations that determine its specificity. We believe that this structural difference extends even to the N-termini of the two PR isoforms, accounting for their contrasting behavior.

Fig. 1. Human Progesterone Receptor test constructs. Constructs were generated, as described in Methods, to test the activity of the individual AFs (one AF), combinations of AFs (two AFs), and the effect of IF on AF activity (ΔIF/ΔAF1). DBD, DNA Binding Domain; HBD, Hormone Binding Domain; NLS, Nuclear Localization Signal; AF1, AF2 and AF3, sites of known transactivation domains; BUS, B Upstream Segment; IF, Intervening Fragment/Inhibitory Function; NT_A and NT_B, N-terminals of A- or B-receptors. Key amino acids are numbered.

Fig. 2. Immunoblot analysis of protein expression. Human PR isoforms and variant constructs were transiently expressed in COS cells, and whole cell extracts were analyzed by either 7.5% (A) or 11.5% (B) SDS-polyacrylamide gel electrophoresis. Immunoblots were probed with a mixture of the A- plus B-specific monoclonal antibody, AB-52, the B-specific monoclonal antibody, B-30, and the anti-DBD polyclonal antibody α266. The latter was generously provided by David Toft (35). This figure is a computer-generated composite of several experiments, in which wild-type hPR_A and/or hPR_B were used as size standards.

Fig. 3. AF autonomous activity is promoter- and cell-specific. CAT transcription levels driven by individual AFs of hPR are shown in HeLa cells (A,B) or T47D-Y cells (C,D). Data shown are for β-galactosidase-normalized extracts from cells that were transfected with PRE_2-$TATA_{tk}$-CAT (A,C) or MMTV-CAT (B,D), and cotransfected with the empty expression vector, pSG5 (lanes 1-4); or expression vectors for hPR_B (lanes 5-8); hPR_A (lanes 9-12); NT_A-DBD (lanes 13,14); DBD-HBD (lanes 15-18); or BUS-DBD (lanes 19,20). DNA concentrations of the expression vectors were 250 ng in HeLa cells, and 1 μg in T47D-Y cells. Transfected cells were left untreated (–) unless the constructs contained an HBD, in which case the cells were treated (+) with 10 nM R5020 for 24 hrs (HeLa cells) or 72 hrs (T47D-Y cells) prior to harvesting. CAT assays were performed as described in the text. The AFs present in each construct are indicated.

Fig. 4. AF3 mediates cooperative interactions with AF1 and AF2 in a promoter-dependent manner. CAT transcription levels driven by constructs containing two or more AFs are shown in HeLa cells (A,B) and T47D-Y cells (C,D). Data shown are for β-galactosidase-normalized extracts from cells that were cotransfected with PRE_2-$TATA_{tk}$-CAT (A,C) or MMTV-CAT (B,D), and with expression vectors for hPR_B (lanes 1-4), BUS-DBD-HBD (lanes 5-8), or NT_B-DBD (lanes 9,10). DNA concentrations for the expression vectors were 250 ng in HeLa cells and 1 μg in T47D-Y cells. Transfected cells were left untreated (–) or were treated with 10 nM R5020 as described. CAT assays were performed as described in the text. The AFs present in each construct are indicated.

Fig. 5. Summary of cell- and promoter-dependent autonomous and cooperative activity among the three AFs of hPR. CAT assays similar to those described in Figures 3 and 4 were quantified by phosphorimaging. Bars represent the average of values derived from six to twenty-five data points for panel A, six to twelve data points for panel B, three to nine data points for panel C, and four to six data points for panel D. CAT activity is shown as a percentage of acetylated [^{14}C]chloramphenicol relative to that produced by hPR_B, which was set at 100%. Standard deviations for transcription levels produced by each construct are indicated, unless the values were too small to graph. *See Figure 6 and Results.

Fig. 6. Transcriptional activity of NT_A-DBD is dependent upon the input DNA concentration. HeLa cells were transfected with 10 to 1000 ng of expression vectors for hPR_B, hPR_A, or NT_A-DBD, and with 2 μg of PRE_2-$TATA_{tk}$-CAT. hPR_B and hPR_A-expressing cells were treated with 10 nM R5020. CAT levels measured by TLC from β-galactosidase-normalized cell extracts, were quantified by phosphorimaging, and expressed graphically as a percentage of acetylated [^{14}C]chloramphenicol. Data points are the average of duplicate assays.

Fig. 7. Removal of IF from hPR_A converts weak into strong transactivators. HeLa cells were transfected with 10, 100 or 1000 ng of A-receptor expression vectors that contain IF (hPR_A) or that lack IF (A-ΔIF), with the B-receptor expression vector (hPR_B); or with the empty vector (pSG5). Transcription from 2 μg of PRE_2-$TATA_{tk}$-CAT was measured as described in Figure 6. Data points represent averages of duplicate samples treated with 10 nM R5020.

Fig. 8. IF represses AF1 and AF2, but not AF3 activity. HeLa cells were cotransfected with 2 μg of PRE_2-$TATA_{tk}$-CAT and 10,100 or 1000 ng of expression vectors for hPR_B (panels A-C), NT_A-DBD plus or minus IF (panel A), DBD-HBD plus or minus IF (panel B) or BUS-DBD plus or minus IF (panel C). The average CAT activity of duplicate assays is shown for each point.

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Figure 1

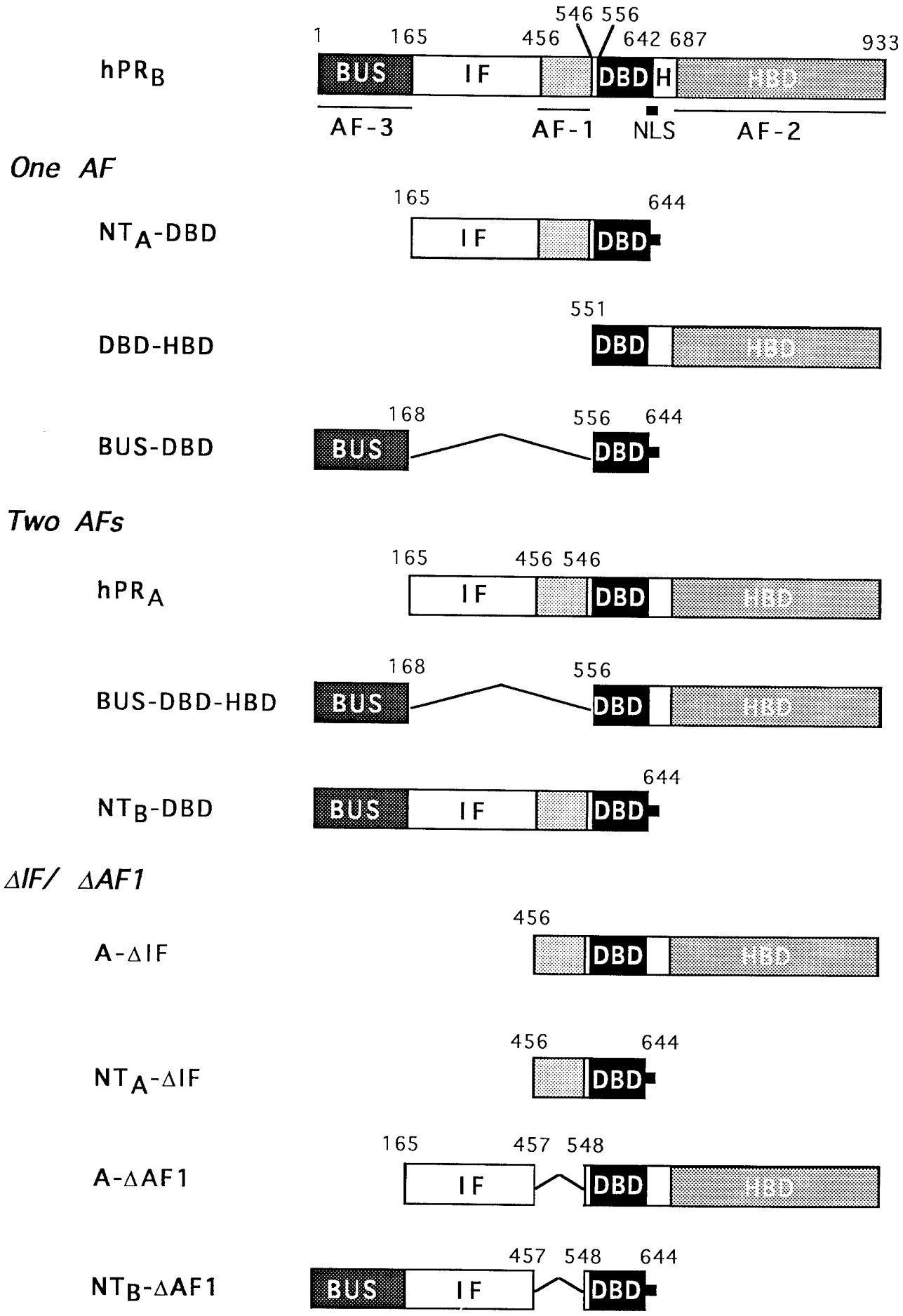
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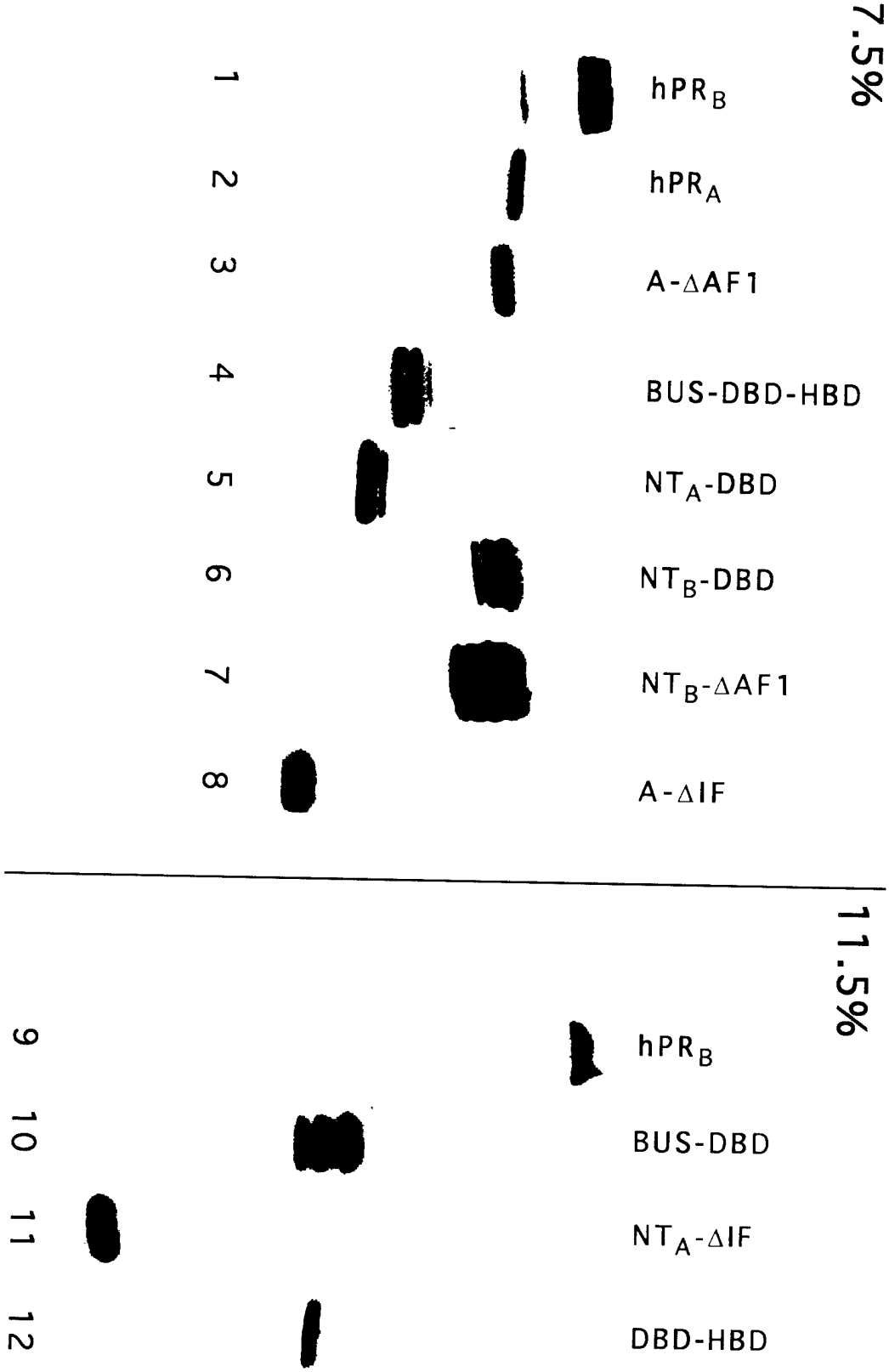
Figure 2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NT$_B$-ΔAF1
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hPR$_B$
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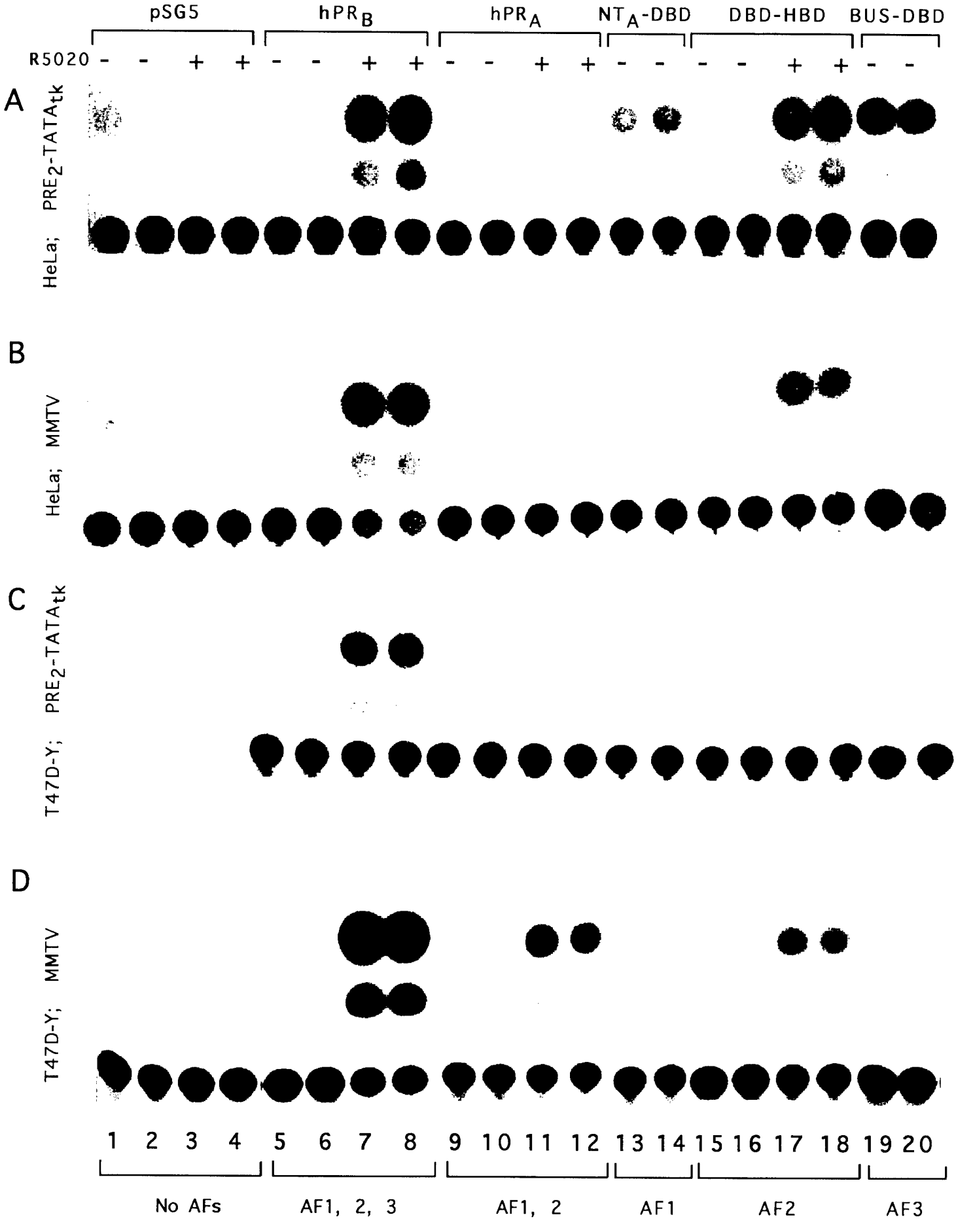
DBD-HBD

Figure 3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hPR$_A$
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DBD-HBD
BUS-DBD
R5020 - - + + - - + + - - + + - - - - + + - -
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HeLa; PRE$_2$-TATA$_{tk}$
B
HeLa; MMTV
C
T47D-Y; PRE$_2$-TATA$_{tk}$
D
T47D-Y; MMTV
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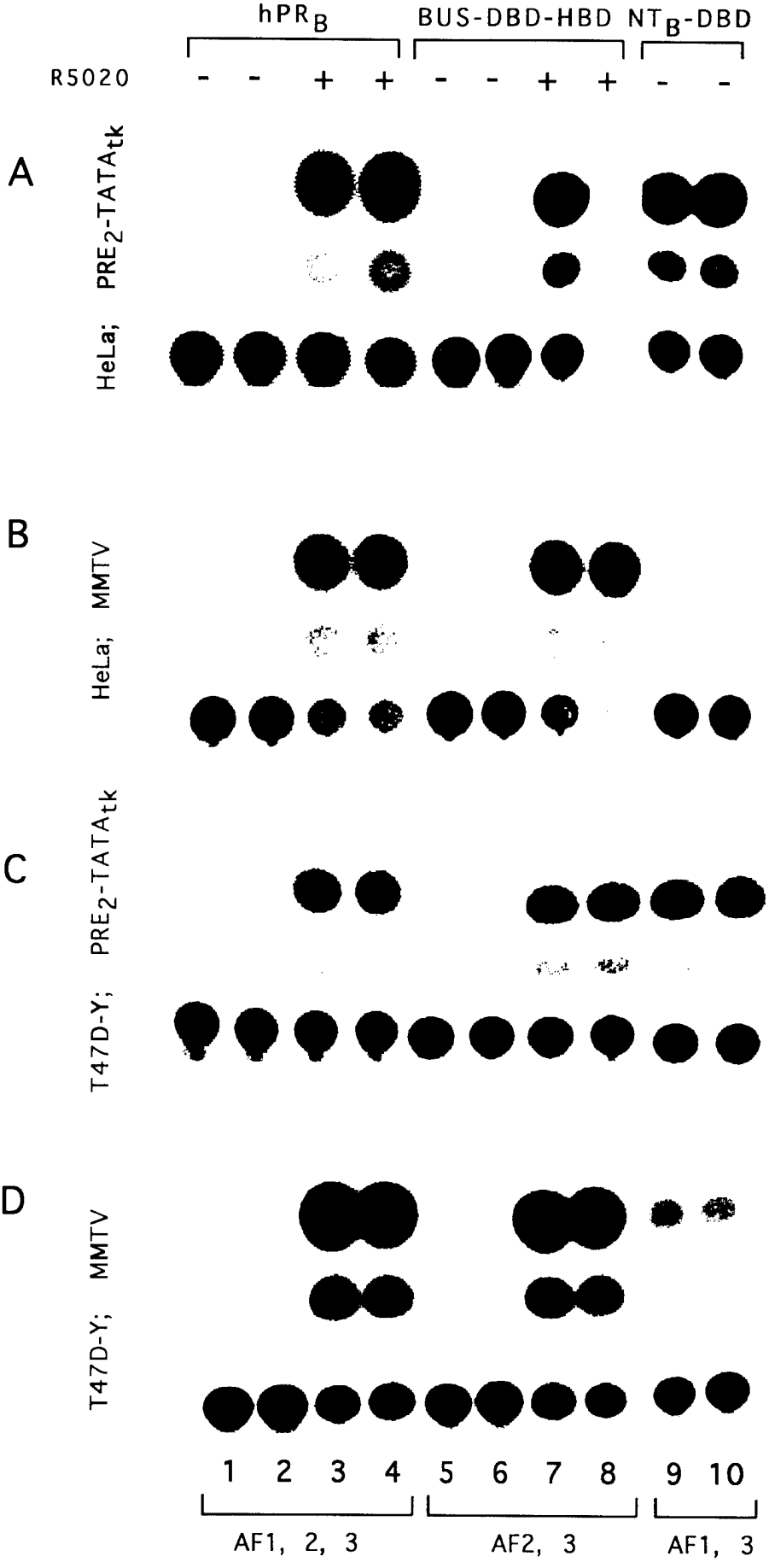
AF1
AF2
AF3

Figure 4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PRE$_2$-TATA$_{tk}$
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B
HeLa; MMTV
C
PRE$_2$-TATA$_{tk}$
T47D-Y;
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T47D-Y; MMTV
1 2 3 4 5 6 7 8 9 10
AF1, 2, 3
AF2, 3
AF1, 3

Figure 5

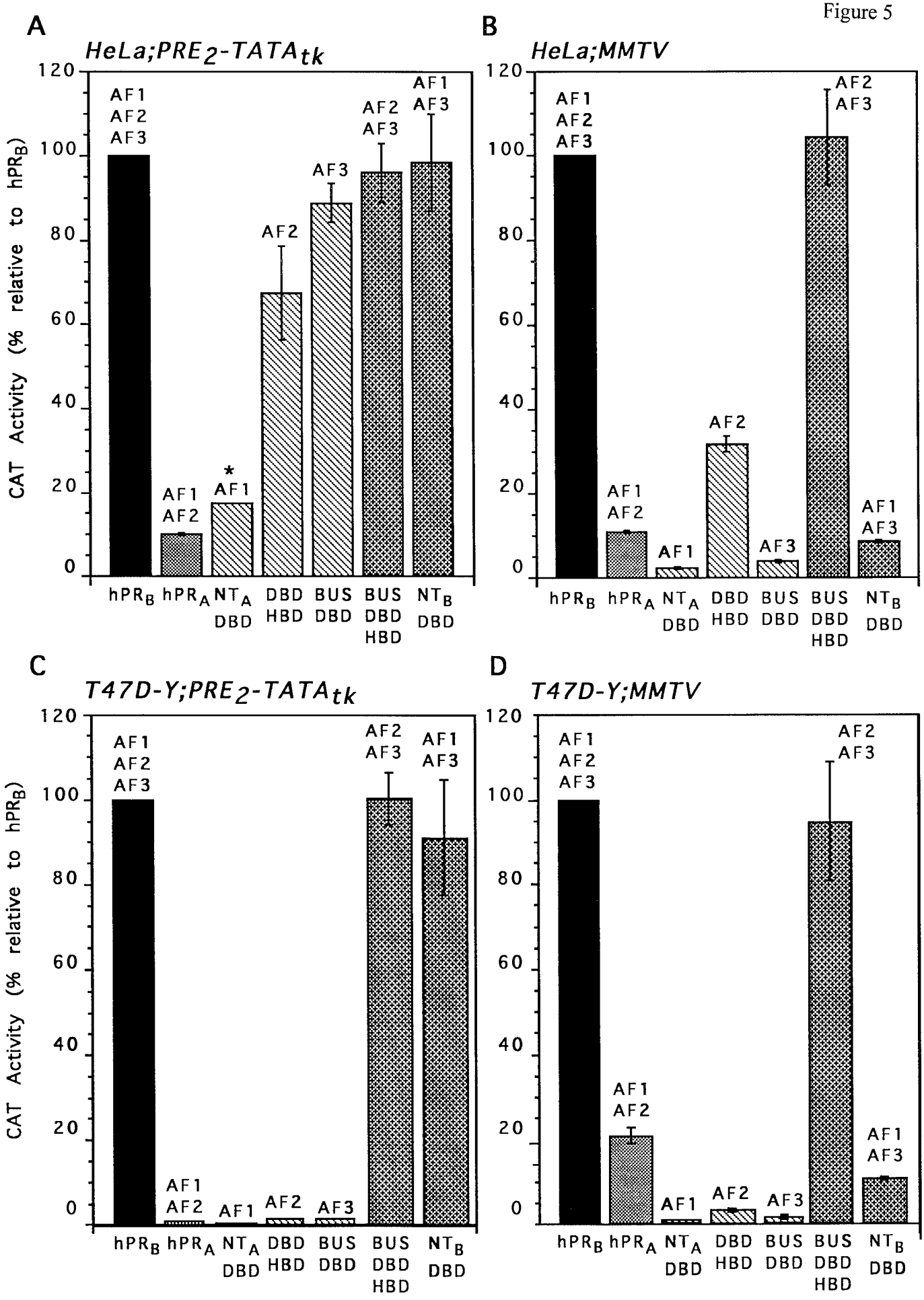

A
HeLa;PRE2-TATAtk
B
HeLa;MMTV
C
T47D-Y;PRE2-TATAtk
D
T47D-Y;MMTV
CAT Activity (% relative to hPRB)
0
20
40
60
80
100
120
AF1 AF2 AF3
AF1 AF2
* AF1
AF2
AF3
AF2 AF3
AF1 AF3
hPRB
hPRA
NTA DBD
DBD HBD
BUS DBD
BUS DBD HBD
NTB DBD

Figure 6

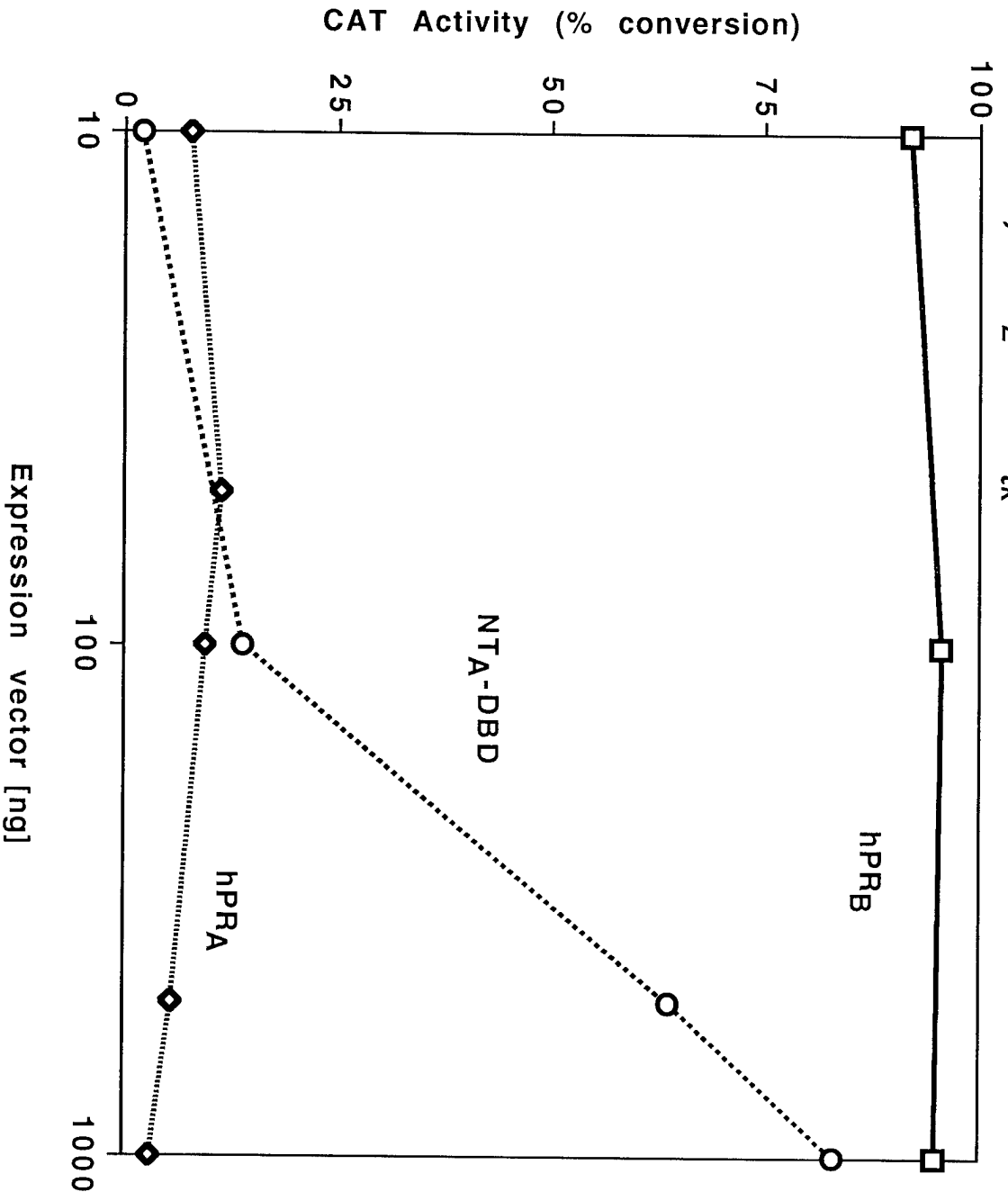

CAT Activity (% conversion)
0
25
50
75
100
HeLa; PRE 2-TATA tk
hPRB
NTA-DBD
hPRA
10
100
1000
Expression vector [ng]

Figure 7

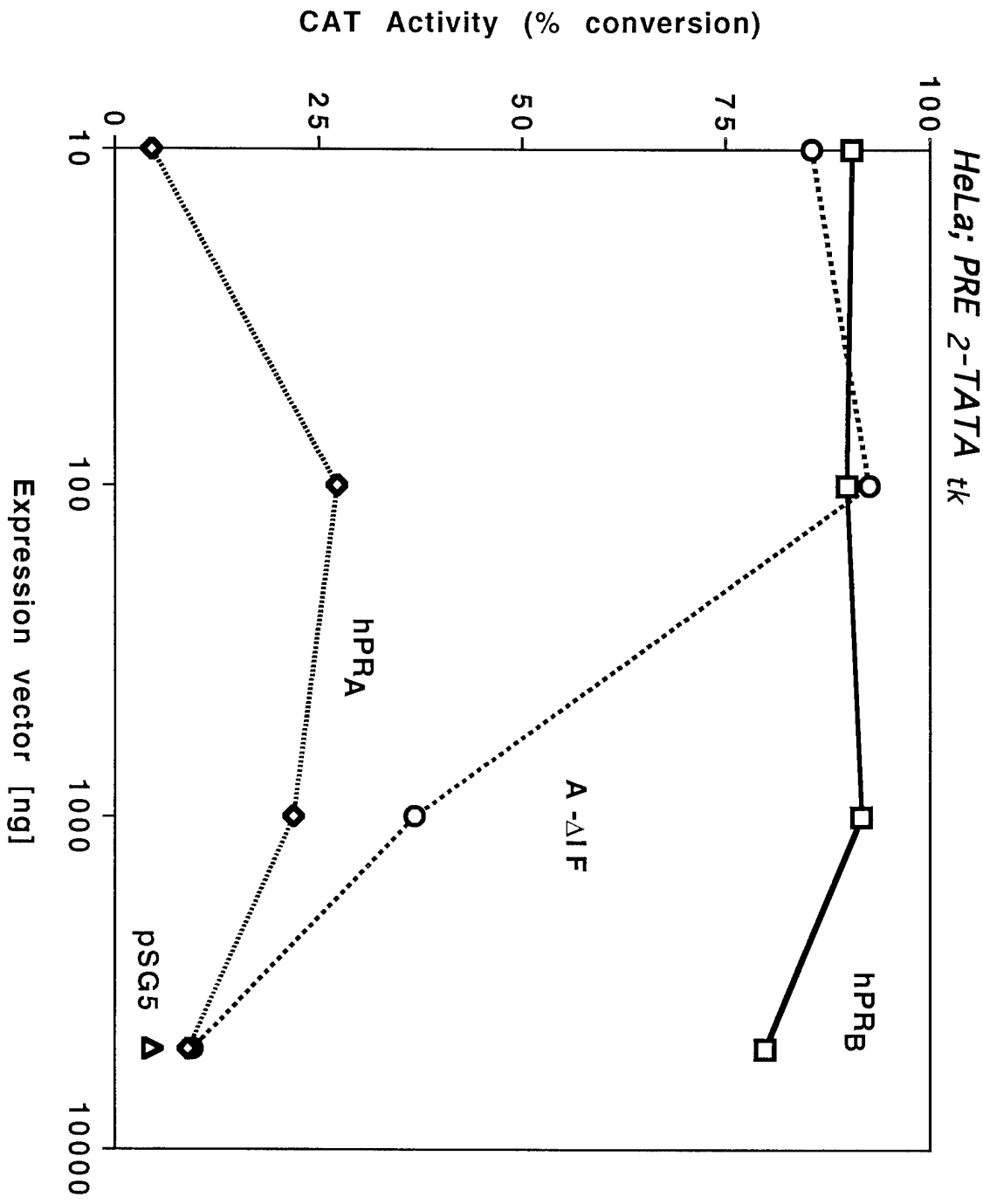
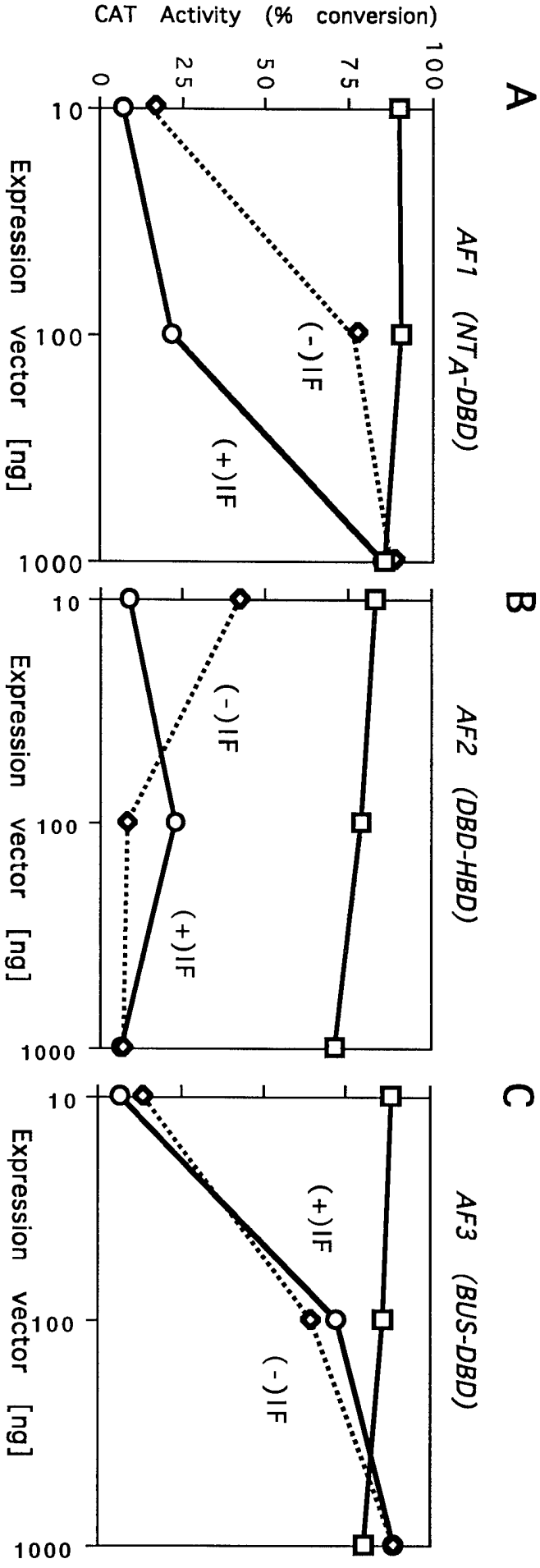

CAT Activity (% conversion)
100
75
50
25
0
HeLa; PRE 2-TATA tk
hPRB
A -ΔIF
hPRA
pSG5
10
100
1000
10000
Expression vector [ng]

Figure 8


CAT Activity (% conversion)
0
25
50
75
100
A
AF1 (NTA-DBD)
(-)IF
(+)IF
B
AF2 (DBD-HBD)
(-)IF
(+)IF
C
AF3 (BUS-DBD)
(+)IF
(-)IF
10
100
1000
Expression vector [ng]